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<b>(21) International Application Number:</b> PCT/US96/05432 <b>(22) International Filing Date:</b> 19 April 1996 (19.04.96)  <b>(30) Priority Data:</b> 08/425,176                      20 April 1995 (20.04.95)                      US 08/425,762                      20 April 1995 (20.04.95)                      US  <b>(71) Applicants:</b> CHIRON VIAGENE, INC. [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US). SYSTEMIX [US/US]; 3155 Porter Drive, Palo Alto, CA 94304 (US).  <b>(72) Inventors:</b> JOLLY, Douglas, J.; 277 Hillcrest Drive, Leucadia, CA 92024 (US). ROBBINS, Joan, M.; 2160 Chalcedony Street, San Diego, CA 92109 (US). KERR, William, G.; 2333 Williams Street, Palo Alto, CA 94306 (US).  <b>(74) Agents:</b> KRUSE, Norman, J. et al.; Chiron Corporation, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA 94662-8097 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> HIGH EFFICIENCY EX VIVO TRANSDUCTION OF HEMATOPOIETIC STEM CELLS BY RECOMBINANT RETROVIRAL PREPARATIONS  <b>(57) Abstract</b> <p>Compositions and methods for the efficient <i>ex vivo</i> introduction of nucleic acid into hematopoietic stem cells mediated by recombinant retrovirus particles is described. The recombinant vector constructs carried by the recombinant retrovirus particles code for the production of a desired gene product having a therapeutic application from a gene of interest. Upon re-introduction into a patient, the transduced hematopoietic stem cells produce a desired gene product in an amount sufficient to treat a particular disease state.</p>		

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**HIGH EFFICIENCY EX VIVO TRANSDUCTION OF  
HEMATOPOIETIC STEM CELLS BY RECOMBINANT  
RETROVIRAL PREPARATIONS**

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**Technical Field**

The present invention relates generally to recombinant retroviruses and gene therapy, and more specifically, to recombinant retroviral particle preparations suitable for a variety of somatic cell gene therapy applications.

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**Background of the Invention**

Since the discovery of DNA in the 1940s and continuing through the most recent era of recombinant DNA technology, substantial research has been undertaken in order to realize the possibility that the course of disease may be affected through interaction with the nucleic acids of living organisms. Most recently, a wide variety of methods have been described for altering or affecting genes of somatic tissue (a process sometimes referred to as "somatic gene therapy"), including for example, viral vectors derived from retroviruses, adenoviruses, vaccinia viruses, herpes viruses, and adeno-associated viruses (*see Jolly, Cancer Gene Therapy* 1(1):51-64, 1994), as well as direct transfer techniques such as lipofection (Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), direct DNA injection (Acsadi *et al.*, *Nature* 352:815-818, 1991), microprojectile bombardment (Williams *et al.*, *PNAS* 88:2726-2730, 1991), liposomes of several types (*see, e.g., Wang et al., PNAS* 84:7851-7855, 1987) and administration of nucleic acids alone (WO 90/11092).

Of these techniques, recombinant retroviral gene delivery methods have been most extensively utilized, in part due to: (1) the efficient entry of genetic material (the vector genome) into replicating cells; (2) an active, efficient process of entry into the target cell nucleus; (3) relatively high levels of gene expression; (4) the potential to target particular cellular subtypes through control of the vector-target cell binding and the tissue-specific control of gene expression; (5) a general lack of pre-existing host immunity; and (6) substantial knowledge and clinical experience which has been gained with such vectors.

Briefly, retroviruses are diploid positive-strand RNA viruses that replicate through an integrated DNA intermediate. In particular, upon infection by the RNA virus, the retroviral genome is reverse-transcribed into DNA by a virally encoded reverse transcriptase that is carried as a protein in each retrovirus. The viral DNA is then integrated pseudo-randomly

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into the host cell genome of the infecting cell, forming a "provirus" which is inherited by daughter cells.

Wild-type retroviral genomes (and their proviral copies) contain three genes (the *gag*, *pol* and *env* genes), which are preceded by a packaging signal ( $\psi$ ), and two long terminal repeat (LTR) sequences which flank both ends. Briefly, the *gag* gene encodes the internal structural (nucleocapsid) proteins. The *pol* gene codes for the RNA-dependent DNA polymerase which reverse transcribes the RNA genome, and the *env* gene encodes the retroviral envelope glycoproteins. The 5' and 3' LTRs contain *cis*-acting elements necessary to promote transcription and polyadenylation of retroviral RNA.

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of retroviral RNA into particles (the *psi* sequence). Removal of the packaging signal prevents encapsidation (packaging of retroviral RNA into infectious virions) of genomic RNA, although the resulting mutant can still direct synthesis of all proteins encoded in the viral genome.

Recombinant retroviruses and various uses thereof have been described in numerous references including, for example, Mann, *et al.* (*Cell* 33:153, 1983), Cane and Mulligan (*Proc. Nat'l. Acad. Sci. USA* 81:6349, 1984), Miller, *et al.*, *Human Gene Therapy* 1:5-14, 1990, U.S. Patent Nos. 4,405,712; 4,861,719; 4,980,289 and PCT Application Nos. WO 89/02,468; WO 89/05,349 and WO 90/02,806). Briefly, a foreign gene of interest may be incorporated into the retrovirus in place of a portion of the normal retroviral RNA. When the retrovirus injects its RNA into a cell, the foreign gene is also introduced into the cell, and may then be integrated into the host's cellular DNA as if it were the retrovirus itself. Expression of this foreign gene within the host results in expression of the foreign protein by the host cell.

One disadvantage, however, of recombinant retroviruses is that they principally infect only replicating cells, thereby making efficient direct gene transfer difficult or impossible for cells characterized as largely non-replicating, such as hematopoietic stem cells. Indeed, some scientists have suggested that other, more efficient methods of gene transfer, such as direct administration of pure plasmid DNA, be utilized (Davis *et al.*, *Human Gene Therapy* 4:733-740, 1993) to introduce nucleic acid molecules into such cells.

In order to increase the efficacy of recombinant retroviruses, the methods which have been suggested have principally been aimed at inducing the desired target cells to replicate, thereby allowing the retroviruses to infect the cells. Such methods have included, for example chemical treatment with 10% carbon tetrachloride in mineral oil (Kaleko, *et al.*, *Human Gene Therapy* 2:27-32, 1991). However, such techniques are not preferred for use

in *ex vivo* techniques designed to introduce nucleic acid molecules encoding therapeutic gene products into mammalian hematopoietic cells.

Mammalian hematopoietic cells provide a diverse range of physiological activities. These cells are divided into lymphoid, myeloid and erythroid lineages. The lymphoid  
5 lineages, comprising B-cells, T-cells, and NK-cells, provides for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes as well as other cells, monitors for the presence of foreign  
10 bodies, provides protection against neoplastic cells, scavenges foreign materials, produces platelets, and the like. The erythroid lineage provides the red blood cells, which act as oxygen carriers.

Despite the diversity of the nature, morphology, characteristics and function of hematopoietic cells, these cells are derived from a single hematopoietic progenitor cell population, termed "stem cells." Stem cells are capable of self-regeneration and may become  
15 lineage restricted progenitors, which further differentiate and expand into specific lineages. As used herein, the term "stem cells" or "hematopoietic stem cells" refers to hematopoietic cells and not stem cells of other cell types. Further, unless indicated otherwise, "stem cells" refers to human hematopoietic stem cells. U.S. Patent No. 5,061,620 describes a substantially homogeneous stem cell composition and the manner of obtaining such a  
20 composition. *See* also the references cited therein.

Stem cells constitute only a small percentage of the total number of hematopoietic cells. Hematopoietic cells are identifiable by the presence of a variety of cell surface  
"markers." Such markers may be either specific to a particular lineage or progenitor cell or be present on more than one cell type. CD34 is a marker found on stem cells and a  
25 significant number of more differentiated progenitor cells. U.S. Patent No. 4,714,680 describes a population of cells expressing the CD34 marker.

Table 1 summarizes probable phenotypes of stem cells in fetal, adult, and mobilized peripheral blood. In Table 1, myelomonocytic stands for myelomonocytic associated markers, NK stands for natural killer cells, FBM and ABM refer to fetal and adult bone  
30 marrow, respectively, and AMPB stands for adult mobilized peripheral blood. As used herein both *infra*, *supra* and in Table 1, the negative sign or, uppercase negative sign, (-) means that the level of the specified marker is undetectable above Ig isotype controls by FACS analysis, and includes cells with very low expression of the specified marker.

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TABLE I

Probable Stem Cell Phenotypes

	NK and T-cell Markers			B-cell Markers			Myelomonocytic			Other						P-gp Activity	
	CD 2	CD 3	CD 8	CD 1 0	CD 1 9	CD 2 0	CD 1 4	CD 1 5	CD 1 6	CD 3 3	CD 3 4	CD 3 8	HLA-DR	C-Ki	Thy	Rbo	
FBM	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	lo	+
ABM	-	-	-	-	-	-	-	-	-	-	+	+	lo/-	+	+	lo	+
AMPB	-	-	-	-	-	-	-	-	-	lo/-	+	+	lo/-	+	+	lo	+

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The ability of stem cells to undergo substantial self-renewal as well as the ability to proliferate and differentiate into all of the hematopoietic lineages makes stem cells the target of choice for a number of gene therapy applications. Successful gene transfer into stem cells should provide long-term repopulation of an individual with the modified cells and their progeny, which will express the desired gene product. By contrast, gene transfer into more mature hematopoietic cells, such as T-cells, at best, provides only transient therapeutic benefit. Thus, there have been world-wide efforts toward finding effective methods of genetically modifying stem cells. For reviews of genetic modification of stem cells *see* Brenner (1993) *J. Hematother.* 2:7-17; Miller (1992) *Nature* 357:455-460; and Nienhuis (1991) *Cancer* 67:2700-2704.

Most efforts to genetically modify stem cells have involved the use of retroviral vectors. Other methods such as liposome-mediated gene transfer or adeno-associated viral vectors have also been used. As discussed previously, retroviral vectors have been the primary vehicle due to the generally high rate of gene transfer obtained in experiments with cell lines, and the ability to obtain stable integration of the genetic material, which ensures that the progeny of the modified cell will contain the transferred genetic material.

Efficient gene transfer into human stem cells has proven difficult due to a variety of factors. Currently used methods of retroviral transduction into human stem cells have a number of practical limitations. One limitation is the extremely low numbers of stem cells present in any tissue. Therefore, in transductions performed with relatively impure

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Efficient gene transfer into human stem cells has proven difficult due to a variety of factors. Currently used methods of retroviral transduction into human stem cells have a number of practical limitations. One limitation is the extremely low numbers of stem cells present in any tissue. Therefore, in transductions performed with relatively impure populations of cells, the ratio of virus particles to stem cells will be quite low. This limitation is compounded by the relatively low titers generally obtained with most retroviral vectors, typically in the range of  $10^5$  to  $10^6$  infectious virions per milliliter. Also, the effect of more differentiated cells in culture on the growth or division of stem cells is not well understood.

In addition, primitive stem cells typically are quiescent in culture; retroviral vectors require target cells to be cycling for stable integration of the retroviral DNA. Cytokines have been used to cause stem cells to cycle, which improves gene transfer efficiency, but the effect of various cytokines in driving stem cells to differentiation remains in question.

The range of host cells that may be infected by a retrovirus or retroviral vector is determined by the viral envelope protein. Therefore, a lack or deficiency of the receptor for the given envelope protein would limit transduction efficiency. In addition, a lack of the requisite cellular factors involved in viral binding, penetration, uncoating of the retroviral vectors, viral replication or integration would limit transduction efficiency.

It is the object of the present invention to provide *ex vivo* methods for using compositions of recombinant retroviral particles to deliver vector constructs encoding genes of interest to hematopoietic stem cells *ex vivo*. The transduced stem cells may then be re-administered to the patient by standard techniques, *e.g.*, intravenous infusion to achieve a desired therapeutic benefit.

### Summary of the Invention

The present invention provides compositions and methods for transducing hematopoietic stem cells. Within one aspect of the invention a method is provided for the production of transduced hematopoietic stem cells comprising obtaining a population of hematopoietic stem cells from a patient and transducing the population of hematopoietic stem

cells with a recombinant retroviral particles substantially free from contamination with replication competent retrovirus, wherein the recombinant retroviral particles carry a vector construct encoding a gene of interest. Within one embodiment of the invention wherein the vector construct encodes a molecule selected from the group consisting of a protein, an active portion of a protein and a RNA molecule with intrinsic biological activity. The protein or active portion of a protein is selected from the group consisting of a cytokine, a colony stimulating factor, a clotting factor, and a hormone. Within another embodiment of the invention methods are provided for treating a genetic disease, cancer, infectious disease, degenerative disease, inflammatory disease, cardiovascular disease, and autoimmune disease by administering to a patient a composition or reintroduction of a therapeutically effective amount of the population of transduced hematopoietic stem cells. In another embodiment the transduced population of hematopoietic stem cells are characterized as CD34<sup>+</sup>Thy-1<sup>+</sup>Lin<sup>-</sup>. In another embodiment the recombinant reroviral vectors used to the transduce the hematopoietic stem cells are xenotropic retroviral vectors. Preferably the reretroviral vector preparations used to transduce the hematopoietic stem cells are high titer preparations. In yet another embodiment the hematopoietic stem cells are expanded *in vitro* prior to re-introduction of the cells into the patient.

In another aspect of the invention an *in vivo* delivery vehicle comprising transplantable hematopoietic stem cells which express a therapeutically effective amount of a gene product encoded by a gene wherein the gene does not occur in hematopoietic stem cells or where the gene occurs in hematopoietic stem cells but is not expressed in the cells and wherein the gene can be modified to be expressed in hematopoietic stem cells is provided.

In other aspects of the invention the transduced hematopoietic stem cells and compositions of hematopoietic stem cells encoding a gene of interest, hematopoietic stem cells transduced with a recombinant xenotropic retroviral vector, and hematopoietic stem cells characterized as CD34<sup>+</sup>Thy-1<sup>+</sup>Lin<sup>-</sup> transduced with recombinant retroviral particles are provided. In one embodiment of this aspect of the invention compositions are provided substantially free from contamination with replication competent retrovirus.

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#### Definition of Terms

The following terms are used throughout the specification. Unless otherwise indicated, these terms are defined as follows:



"Event-specific promoter" refers to transcriptional promoter/enhancer or locus defining elements, or other elements which control gene expression as discussed above, whose transcriptional activity is altered upon response to cellular stimuli. Representative examples of such event-specific promoters include thymidine kinase or thymidylate synthases promoters, alpha or beta interferon promoters and promoters that respond to the presence of hormones (either natural, synthetic or from other non-host organisms).

"Tissue-specific promoter" refers to transcriptional promoter/enhancer or locus defining elements, or other elements which control gene expression as discussed above, which are preferentially active in a limited number of hematopoietic tissue types. Representative examples of such hematopoietic tissue-specific promoters include, but are not limited to, the IgG promoter,  $\alpha$ - or  $\beta$ -globin promoters, T-cell receptor promoter, Granzyme A, Granzyme B, CD8, and CD11b.

"Transduction" involves the association of a replication defective, recombinant retroviral particle with a cellular receptor, followed by introduction of the nucleic acids carried by the particle into the cell. "Transfection" refers to a method of physical gene transfer wherein no retroviral particle is employed.

"Vector construct", "retroviral vector", "recombinant vector", and "recombinant retroviral vector" refer to a nucleic acid construct capable of directing the expression of a gene of interest. The retroviral vector must include at least one transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by other means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Such vector constructs must also include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present in the retroviral vector). Optionally, the vector construct may also include a signal which directs polyadenylation, as well as one or more restriction sites and a translation termination sequence. By way example, such vectors will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR or a portion thereof. In order to express a desired gene product from such a vector, a gene of interest encoding the desired gene product is also included.

Numerous aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following detailed description which provides illumination of the practice of the invention.

### Detailed Description of the Invention

5 The present invention is based on the unexpected discovery that recombinant retroviral particles carrying a vector construct comprising a gene of interest can be used *ex vivo* to efficiently transduce hematopoietic stem cells. As a result, recombinant retroviral particles according to the invention can be used for purposes of somatic gene therapy. A more thorough description of such recombinant retroviral particles, their production and packaging, and uses therefore is provided below.

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### Generation of Recombinant Retroviral Vectors

As noted above, the present invention provides compositions and methods comprising recombinant retroviral particles, including recombinant xenotropic retroviral  
15 vector particles, for use in *ex vivo* somatic gene therapy. The construction of recombinant retroviral vectors and particles is described in greater detail in an application entitled "Recombinant Retroviruses". Production of transduction competent recombinant xenotropic retroviral particles is described in U.S.S.N. 08/156,789, and U.S.S.N. 07/965,084, which are hereby incorporated by reference in their entirety. In general, the recombinant vector  
20 constructs described herein are prepared by selecting a plasmid with a strong promoter, and appropriate restriction sites for insertion of DNA sequences of interest downstream from the promoter.

According to the invention, the recombinant vector construct is carried by a recombinant retrovirus. Retroviruses are RNA viruses with a single positive strand genome  
25 which in general, are nonlytic. Upon infection, the retrovirus reverse transcribes its RNA into DNA, forming a provirus which is inserted into the host cell genome. The retroviral genome can be divided conceptually into two parts. The "trans-acting" portion consists of the region coding for viral structural proteins, including the group specific antigen (*gag*) gene for synthesis of the core coat proteins; the *pol* gene for the synthesis of the reverse transcriptase and integrase enzymes; and the envelope (*env*) gene for the synthesis of  
30 envelope glycoproteins. The "cis-acting" portion consists of regions of the genome that is finally packaged into the viral particle. These regions include the packaging signal, long terminal repeats (LTR) with promoters and polyadenylation sites, and two start sites for DNA replication. The internal or "trans-acting" part of the cloned provirus is replaced by the  
35 gene of interest to create a "vector construct". When the vector construct is placed into a

cell where viral packaging proteins are present, the transcribed RNA will be packaged as a viral particle which, in turn, will bud off from the cell. These particles are used to transduce tissue cells, allowing the vector construct to integrate into the cell genome. Although the vector construct expresses its gene product, the virus carrying it is replication defective because the trans-acting portion of the viral genome is absent. Various assays may be utilized in order to detect the presence of any replication competent infectious retrovirus. One preferred assay is the extended S<sup>+</sup>L<sup>-</sup> assay described in Example 4.

In the broadest terms, the retroviral vectors of the invention comprise a transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by other means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Such vector constructs must also include a packaging signal, long terminal repeats (LTRs) or portion thereof, and positive and negative strand primer binding sites appropriate to the retrovirus used (if these are not already present in the retroviral vector). Optionally, the vector construct may also include a signal which directs polyadenylation, as well as one or more restriction sites and a translation termination sequence. By way example, such vectors will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis, and a 3' LTR or a portion thereof. Such vectors do not contain one or more of a complete *gag*, *pol*, or *env* gene, thereby rendering them replication incompetent. In addition, nucleic acid molecules coding for a selectable marker are neither required nor preferred.

Preferred retroviral vectors contain a portion of the *gag* coding sequence, preferably that portion which comprises a splice donor and splice acceptor site, the splice acceptor site being positioned such that it is located adjacent to and upstream from the gene of interest. In a particularly preferred embodiment, the *gag* transcriptional promoter is positioned such that an RNA transcript initiated therefrom contains the 5' *gag* LTR and the gene of interest. As an alternative to the *gag* promoter to control expression of the gene of interest, other suitable promoters, some of which are described below, may be employed. In addition, alternate enhancers may be employed in order to increase the level of expression of the gene of interest.

In preferred embodiments of the invention, retroviral vectors are employed, particularly those based on Moloney murine leukemia virus (MoMLV). MoMLV is a murine retrovirus which has poor infectivity outside of mouse cells. The related amphotropic N2 retrovirus will infect cells from human, mouse and other organisms. Other preferred retroviruses which may be used in the practice of the present invention include gibbon ape

leukemia virus (GALV) (Todaro, *et al*, *Virology*, 67:335, 1975; Wilson, *et al*, *J. Vir.*, 63:2374, 1989), feline immunodeficiency virus (FIV) (Talbutt, *et al*, *Proc. Nat'l. Acad. Sci. USA*, 86:5743, 1984), and feline leukemia virus (FeLV) (Leprevette, *et al*, *J. Vir.*, 50:884, 1984; Elder, *et al.*, *J. Vir.*, 46:871, 1983; Steward, *et al.*, *J. Vir.*, 58:825, 1986; Riedel, *et al.*, *J. Vir.*, 60:242, 1986), although retroviral vectors according to the invention derived from other type C retroviruses (Weiss, *RNA Tumor Viruses*, vols. I and II, Cold Spring Harbor Laboratory Press, N.Y.) can also be generated.

A variety of promoters can be used in the vector constructs of the invention, including but not necessarily limited to the cytomegalovirus major immediate early promoter (CMV MIE), the early and late SV40 promoters, the adenovirus major late promoter, thymidine kinase or thymidylate synthase promoters, alpha or beta interferon promoters, event or tissue specific promoters, *etc.* Promoters may be chosen so as to potentially drive high levels of expression or to produce relatively weak expression, as desired. As those in the art will appreciate, numerous RNA polymerase II and RNA polymerase III dependent promoters can be utilized in practicing the invention.

In one embodiment, recombinant retroviral vectors comprising a gene of interest are under the transcriptional control of an event-specific promoter, such that upon activation of the event-specific promoter the gene is expressed. Numerous event-specific promoters may be utilized within the context of the present invention, including for example, promoters which are activated by cellular proliferation (or are otherwise cell-cycle dependent) such as the thymidine kinase or thymidylate synthase promoters (Merrill, *Proc. Natl. Acad. Sci. USA*, 86:4987, 1989; Deng, *et al.*, *Mol. Cell. Biol.*, 9:4079, 1989); or the transferrin receptor promoter, which will be transcriptionally active primarily in rapidly proliferating cells (such as hematopoietic cells) which contain factors capable of activating transcription from these promoters preferentially to express gene products from gene of interest; promoters such as the alpha or beta interferon promoters which are activated when a cell is infected by a virus (Fan and Maniatis, *EMBO J.*, 8:101, 1989; Goodbourn, *et al.*, *Cell*, 45:601, 1986); and promoters which are activated by the presence of hormones, *e.g.*, estrogen response promoters. See Toohey *et al.*, *Mol. Cell. Biol.*, 6:4526, 1986; and promoters that are activated in response to cellular stress or insult, *e.g.*, electrophilic response elements (Friling, *et al.*, *PNAS*, 87:6258, 1990)

In another embodiment, recombinant retroviral vectors are provided which comprise a gene of interest under the transcriptional control of a tissue-specific promoter, such that upon activation of the tissue-specific promoter the gene is expressed. A wide variety of tissue-specific promoters may be utilized within the context of the present invention

Representative examples of such promoters include: B-cell specific promoters such as the IgG promoter; T-cell specific promoters such as the T-cell receptor promoter (Anderson, *et al.*, *Proc. Natl. Acad. Sci. USA*, 85:3551, 1988; Winoto and Baltimore, *EMBO J.*, 8:29, 1989); bone-specific promoters such as the osteocalcin promoter (Markose, *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1701, 1990; McDonnell, *et al.*, *Mol. Cell. Biol.*, 9:3517, 1989; Kerner, *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:4455, 1989), the IL-2 promoter, IL-2 receptor promoter, and the MHC Class II promoter, and hematopoietic tissue specific promoters, for instance erythroid specific-transcription promoters which are active in erythroid cells, such as the porphobilinogen deaminase promoter (Mignotte, *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:6458, 1990),  $\alpha$ - or  $\beta$ -globin specific promoters (van Assendelft, *et al.*, *Cell*, 56:969, 1989; Forrester, *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:5439, 1989), endothelial cell specific promoters such as the vWf promoter, megakaryocyte specific promoters such as  $\beta$ -thromboglobulin, and many other tissue-specific promoters. Examples of promoters that may be used to cause expression of the introduced sequence in specific cell types include Granzyme A and Granzyme B for expression in T-cells and NK cells, the CD34 promoter for expression in stem and progenitor cells, the CD8 promoter for expression in cytotoxic T-cells, and the CD11b promoter for expression in myeloid cells.

Retroviral vectors according to the invention may also contain a non-LTR enhancer or promoter, *e.g.*, a CMV or SV40 enhancer operably associated with other elements employed to regulate expression of the gene of interest. Additionally, retroviral vectors from which the 3' LTR enhancer has been deleted, thereby inactivating the 5' LTR upon integration into a host cell genome, are also contemplated by the invention. A variety of other elements which control gene expression may also be utilized within the context of the present invention, including, for example, locus-defining elements such as those from the  $\beta$ -globin gene and CD2, a T-cell marker. In addition, elements which control expression at the level of splicing, nuclear export, and/or translation may also be included in the retroviral vectors. Representative examples include the  $\beta$ -globin intron sequences, the *rev* and *rre* elements from HIV-1, the constitutive transport element (CTE) from Mason-Pfizer monkey virus (MPMV), a 219 nucleotide sequence that allows *rev*-independent replication of *rev*-negative HIV proviral clones, and a Kozak sequence. Rev protein functions to allow nuclear export of unspliced and singly spliced HIV RNA molecules. The MPMV element allows nuclear export of intron-containing mRNA. The CTE element maps to MPMV nucleotides 8,022-8,240 (Bray, *et al.*, *Biochemistry*, 91:1256, 1994).

In another preferred embodiment, the retroviral vector contains a splice donor (SD) site and a splice acceptor (SA) site, wherein the SA is located upstream of the site where the

gene of interest is inserted into the recombinant retroviral vector. In a preferred embodiment, the SD and SA sites will be separated by a short, *i.e.*, less than 400 nucleotide, intron sequence. Such sequences may serve to stabilize RNA transcripts. Such stabilizing sequences typically comprise a SD-intron-SA configuration located 5' to the gene of interest.

5       The recombinant retroviral vectors of the invention will also preferably contain transcriptional promoters derived from the *gag* region operably positioned such that a resultant transcript comprising the gene of interest further comprises a 5' *gag* LTR (untranslated region) upstream of the gene of interest.

10       The present invention also provides for multivalent vector constructs, the construction of which may require two promoters when two proteins are being expressed, because one promoter may not ensure adequate levels of gene expression of the second gene. In particular, where the vector construct expresses an antisense message or ribozyme, a second promoter may not be necessary. Within certain embodiments, an internal ribosome binding site (IRBS) or herpes simplex virus thymidine kinase (HSVTK) promoter is placed in  
15       conjunction with the second gene of interest in order to boost the levels of gene expression of the second gene. Briefly, with respect to IRBS, the upstream untranslated region of the immunoglobulin heavy chain binding protein has been shown to support the internal engagement of a bicistronic message (Jacejak, *et al.*, *Nature* 353:90, 1991). This sequence is small, approximately 300 base pairs, and may readily be incorporated into a vector in order  
20       to express multiple genes from a multi-cistronic message whose cistrons begin with this sequence.

      Retroviral vector constructs according to the invention will often be encoded on a plasmid, a nucleic acid molecule capable of propagation, segregation, and extra-chromosomal maintenance upon introduction into a host cell. As those in the art will  
25       understand, any of a wide range of existing or new plasmids can be used in the practice of the invention. Such plasmids contain an origin of replication and typically are modified to contain a one or more multiple cloning sites to facilitate recombinant use. Preferably, plasmids used in accordance with the present invention will be capable of propagation in both eukaryotic and prokaryotic host cells.

### Generation of Packaging Cells

Another aspect of the invention relates to methods of producing recombinant retroviral particles incorporating the retroviral vectors described herein. In one embodiment, vectors are packaged into infectious virions through the use of a packaging cell. Briefly, a packaging cell is a cell comprising, in addition to its natural genetic complement, additional nucleic acids coding for those retroviral structural polypeptides required to package a retroviral genome, be it recombinant (*i.e.*, a retroviral vector) or otherwise. The retroviral particles are made in packaging cells by combining the retroviral genome with a capsid and envelope to make a transduction competent, preferably replication defective, virion. Briefly, these and other packaging cells will contain one, and preferably two or more nucleic acid molecules coding for the various polypeptides, *e.g.*, *gag*, *pol*, and *env*, required to package a retroviral vector into an infectious virion. Upon introduction of a nucleic acid molecule coding for the retroviral vector, the packaging cells will produce infectious retroviral particles. Packaging cell lines transfected with a retroviral vector according to the invention which produce infectious virions are referred to as "producer" cell lines.

A wide variety of animal cells may be utilized to prepare the packaging cells of the present invention, including without limitation, epithelial cells, fibroblasts, hepatocytes, endothelial cells, myoblasts, astrocytes, lymphocytes, *etc.*. Preferentially, cell lines are selected that lack genomic sequences which are homologous to the retroviral vector construct, *gag/pol* expression cassette and *env* expression cassette to be utilized. Methods for determining homology may be readily accomplished by, for example, hybridization analysis (Martin *et al.*, *Proc. Natl. Acad. Sci., USA*, 78:4892, 1981; and U.S.S.N. 07/800,921, *supra*).

The most common packaging cell lines (PCLs) used for MoMLV vector systems (psi2, PA12, PA317) are derived from murine cell lines. However, murine cell lines are typically not the preferred choice to produce retroviral vectors intended for human therapeutic use because such cell lines are known to: contain endogenous retroviruses, some of which are closely related in sequence and retroviral type to the MLV vector system preferred for use in practicing the present invention; contain non-retroviral or defective retroviral sequences that are known to package efficiently; and cause deleterious effects due to the presence of murine cell membrane components.

An important consideration in developing packaging cell lines useful in the invention is the production therefrom of replication incompetent virions, or avoidance of generating replication-competent retrovirus (RCR) (Munchau, *et al.*, *Virology*, 176:262, 1991). This

will ensure that infectious retroviral particles harboring the recombinant retroviral vectors of the invention will be incapable of independent replication in target cells, be they *in vitro* or *in vivo*. Independent replication, should it occur, may lead to the production of wild-type virus, which in turn could lead to multiple integrations into the chromosome(s) of a patient's cells, thereby increasing the possibility of insertional mutagenesis and its associated problems.

5 RCR production can occur in at least two ways: (1) through homologous recombination between the therapeutic proviral DNA and the DNA encoding the retroviral structural genes ("gag/pol" and "env") present in the packaging cell line; and (2) generation of replication-competent virus by homologous recombination of the proviral DNA with the very large

10 number of defective endogenous proviruses found in murine packaging cell lines.

To circumvent inherent safety problems associated with the use of murine based recombinant retroviruses, as are preferred in the practice of this invention, packaging cell lines may be derived from various non-murine cell lines. These include cell lines from various mammals, including humans, dogs, monkeys, mink, hamsters, and rats. As those in

15 the art will appreciate, a multitude of packaging cell lines can be generated using techniques known in the art (for instance, see U.S.S.N. 08/156,789 and U.S.S.N. 08/136,739). In preferred embodiments, cell lines are derived from canine or human cell lines, which are known to lack genomic sequences homologous to that of MoMLV by hybridization analysis (Martin *et al.*, *supra*). A particularly preferred parent dog cell line is D17 (ATCC. CRL

20 8543). HT-1080 (ATCC. CCL 121; Graham *et al.*, *Vir.*, 52:456, 1973) and 293 cells (Felgner *et al.*, *Proc. Nat'l. Acad. Sci. USA* 84:7413, 1987) represent particularly preferred parental human cell lines. Construction of packaging cell lines from these cell lines for use in conjunction with a MoMLV based recombinant retroviral vector is described in detail in U.S.S.N. 08/156,789, *supra*.

25 Thus, a desirable prerequisite for the use of retroviruses in gene therapy is the availability of retroviral packaging cell lines incapable of producing replication competent, or "wild-type," virus. As packaging cell lines contain one or more nucleic acid molecules coding for the structural proteins required to assemble the retroviral vector into infectious retroviral particles, recombination events between these various constructs might produce

30 replication competent virus, *i.e.*, infectious retroviral particles containing a genome encoding all of the structural genes and regulatory elements, including a packaging signal, required for independent replication. In the past several years, many different constructions have been developed in an attempt to obviate this concern. Such constructions include: deletions in the 3' LTR and portions of the 5' LTR (see, Miller and Buttimore, *Mol. Cell. Biol.*, 6:2895,

35 1986), where two recombination events are necessary to form RCR; use of complementary



portions of helper virus, divided among two separate plasmids, one containing *gag* and *pol*, and the other containing *env* (see, Markowitz *et al.*, *J. Virol.*, 62:1120; and Markowitz *et al.*, *Virology*, 167: 600, 1988), where three recombination events are required to generate RCR.

The ability to express *gag/pol* and *env* function separately allows for manipulation of these functions independently. A cell line that expresses ample amounts of *gag/pol* can be used, for example, to address questions of titer with regard to *env*. ~~One factor resulting in~~ measured low titers is the density of appropriate receptor molecules on the target cell or tissue. A second factor is the affinity of the receptor for the retroviral envelope protein. One report suggests that xenotropic vector, in the presence of replication-complement virus, may more effectively infect human hematopoietic progenitor cells (Eglitis, *et al.*, *Biochem. Biophys. Res. Comm.* 151:201, 1988). vector-containing particles, in the presence of replication-competent xenotropic virus, also infect cells from other species which are not easily infectable by amphotropic virus such as bovine, porcine, and equine cells (Delouis, *et al.*, *Biochem. Biophys. Res. Comm.* 169:80, 1990). In a preferred embodiment of the invention, packaging cell lines which express a xenotropic *env* gene are provided. Significantly, recombinant xenotropic retroviral particles produced from such packaging cell lines are substantially free from association with replication competent retrovirus ("RCR").

More recently, further improved methods and compositions for inhibiting the production of replication incompetent retrovirus have been developed. See co-owned U.S.S.N. 09/028,126, filed September 7, 1994. Briefly, the spread of replication competent retrovirus generated through recombination events between the recombinant retroviral vector and one or more of the nucleic acid constructs coding for the retroviral structural proteins may be prevented by providing vectors which encode a non-biologically active inhibitory molecule, but which produce a nucleic acid molecule encoding a biologically active inhibitory molecule in the event of such recombination. The expression of the inhibitory molecule prevents production of RCR either by killing the producer cell(s) in which that event occurred or by suppressing production of the retroviral vectors therein. A variety of inhibitory molecules may be used, including ribozymes, which cleave the RNA transcript of the replication competent virus, or a toxin such as ricin A, tetanus, or diphtheria toxin, herpes thymidine kinase, *etc.* As those in the art will appreciate, the teachings therein may be readily adapted to the present invention.

In addition to issues of safety, the choice of host cell line for the packaging cell line is of importance because many of the biological properties (such as titer) and physical properties (such as stability) of retroviral particles are dictated by the properties of the host cell. For instance, the host cell must efficiently express (transcribe) the vector RNA genome.

prime the vector for first strand synthesis with a cellular tRNA, tolerate and covalently modify the MLV structural proteins (proteolysis, glycosylation, myristylation, and phosphorylation), and enable virion budding from the cell membrane. For example, it has been found that vector made from the mouse packaging line PA317 is retained by a 0.3 micron filter, while that made from a CA line will pass through. Furthermore, sera from primates, including humans, but not that from a wide variety of lower mammals or birds, is known to inactivate retroviruses by an antibody independent complement lysis method. Such activity is non-selective for a variety of distantly related retroviruses. Retroviruses of avian, murine (including MoMLV), feline, and simian origin are inactivated and lysed by normal human serum. See Welsh, *et al.*, *Nature*, 257:612, 1975; Welsh, *et al.*, *Virology*, 74:432, 1976; Banapour, *et al.*, *Virology*, 152:268, 1986; and Cooper, *et al.*, (1986) *Immunology of the Complement System*, Pub. American Press, Inc., pp:139-162. In addition, replication competent murine amphotropic retroviruses injected intravenously into primates *in vivo* are cleared within 15 minutes by a process mediated in whole or in part by primate complement (Cornetta, *et al.*, *Human Gene Therapy*, 1:15, 1990; Cornetta, *et al.*, *Human Gene Therapy*, 2:5, 1991). However, it has recently been discovered that retroviral resistance to complement inactivation by human serum is mediated, at least in some instances, by the packaging cell line from which the retroviral particles were produced. Retroviruses produced from various human packaging cell lines were resistant to inactivation by a component of human serum, presumably complement, but were sensitive to serum from baboons and macaques. See commonly owned U.S.S.N. 08/367,071, filed on December 30, 1994. Thus, in a preferred embodiment of the invention, recombinant retroviral particles are produced in human packaging cell lines, with packaging cell lines derived from HT1080 or 293 cells being particularly preferred.

In addition to generating infectious, replication defective recombinant retroviruses as described above, at least two other alternative systems can be used to produce recombinant retroviruses carrying the vector construct. One such system (Webb, *et al.*, *BBRC*, 190:536, 1993) employs the insect virus, baculovirus, while the other takes advantage of the mammalian viruses vaccinia and adenovirus (Pavirani, *et al.*, *BBRC*, 145:234, 1987). Each of these systems can make large amounts of any given protein for which the gene has been cloned. For example, see Smith, *et al.* (*Mol. Cell. Biol.*, 3:12, 1983); Piccini, *et al.* (*Meth. Enzymology*, 153:545, 1987); and Mansour *et al.* (*Proc. Natl. Acad. Sci. USA*, 82:1359, 1985). These retroviral vectors can be used to produce proteins in tissue culture cells by insertion of appropriate genes and, hence, could be adapted to make retroviral vector particles from tissue culture. In an adenovirus system, genes can be inserted into vectors and

used to express proteins in mammalian cells either by *in vitro* construction (Ballay, *et al.*, 4:3861, 1985) or by recombination in cells (Thummel, *et al.*, *J. Mol. Appl. Genetics*, 1:435, 1982).

5 An alternative approach involves cell-free packaging systems. For instance, retroviral structural proteins can be made in a baculovirus system (or other protein production systems, such as yeast or *E. coli*) in a similar manner as described in Smith *et al.* (*supra*). Recombinant retroviral genomes are made by *in vitro* RNA synthesis (*see*, for example, Flamant and Sorge, *J. Virol.*, 62:1827, 1988). The structural proteins and RNA genomes are then mixed with tRNA, followed by the addition of liposomes with embedded *env* protein  
10 and cell extracts (typically from mouse cells) or purified components (which provide *env* and other necessary processing, and any or other necessary cell-derived functions). The mixture is then treated (*e.g.*, by sonication, temperature manipulation, or rotary dialysis) to allow encapsidation of nascent retroviral particles. This procedure allows production of high titer, replication incompetent recombinant retroviruses without contamination with pathogenic  
15 retroviruses or replication-competent retroviruses.

Another important factor to consider in the selection of a packaging cell line is the viral titer produced therefrom following introduction of a nucleic acid molecule from which the retroviral vector is produced. Many factors can limit viral titer. One of the most significant limiting factors is the expression level of the packaging proteins *gag*, *pol*, and *env*.  
20 In the case of retroviral particles, expression of retroviral vector RNA from the provirus can also significantly limit titer. In order to select packaging cells and the resultant producer cells expressing high levels of the required products, an appropriate titering assay is required. As described in greater detail below, a suitable PCR-based titering assay can be utilized.

In addition to preparing packaging and producer cell lines which supply proteins for  
25 packaging that are homologous for the backbone of the viral vector, *e.g.*, retroviral *gag*, *pol*, and *env* proteins for packaging of a retroviral vector, packaging and producer systems which result in chimeric viral particles, for instance a MoMLV-based retroviral vector packaged in a DNA virus capsid, may also be employed. Many other packaging and producer systems based on viruses unrelated to that of the viral vector can also be utilized, as those in the art  
30 will appreciate.

#### Altering the Host Range of Recombinant Retroviral Particles

Another aspect of the invention concerns recombinant xenotropic retroviral particles  
35 which have an altered host range as compared to retroviral particles containing amphotropic

envelope proteins. The host cell range specificity of a retrovirus is determined in part by the *env* gene products present in the lipid envelope. Interestingly, envelope proteins from one retrovirus can often substitute, to varying degrees, for that of another retrovirus, thereby altering host range of the resultant vector. Thus, packaging cell lines (PCLs) have been

5 generated to express either amphotropic, ecotropic, xenotropic, polytropic, or other envelope tropisms. Additionally, retroviruses according to the invention which contain "hybrid" or "chimeric" xenotropic envelope proteins can be similarly generated. Retroviral particles produced from any of these packaging cell lines can be used to infect any cell which contains the corresponding distinct receptor (Rein and Schultz, *Virology*, 136:144, 1984).

10 The assembly of retroviruses is characterized by selective inclusion of the retroviral genome and accessory proteins into a budding retroviral particle. Interestingly, envelope proteins from non-murine retrovirus sources can be used for pseudotyping (*i.e.*, the encapsidation of viral RNA from one species by viral proteins of another species) a vector to alter its host range. Because a piece of cell membrane buds off to form the retroviral

15 envelope, molecules normally in the membrane may be carried along on the viral envelope. Thus, a number of different potential ligands can be put on the surface of retroviral particles by manipulating the packaging cell line in which the vectors are produced or by choosing various types of cell lines with particular surface markers.

Briefly, in this aspect the present invention provides for enveloped retroviral particles

20 comprising: a nucleocapsid including nucleocapsid protein having an origin from a first virus, which is a retrovirus; a packagable nucleic acid molecule encoding a gene of interest associated with the nucleocapsid; and a membrane-associated xenotropic protein which determines a host range.

In another preferred form of the present invention, the membrane-associated

25 xenotropic protein of the vector particles is a chimeric or hybrid protein including an exterior receptor binding domain and a membrane-associated domain from a xenotropic envelope protein, at least a portion of the exterior receptor binding domain being derived from a different origin than at least a portion of the membrane-associated domain. The chimeric protein is preferably derived from two origins, wherein no more than one of the two origins

30 is retroviral.

Another embodiment of this aspect of the present invention concerns cell lines that produce the foregoing vector particles. Preferably, such cell lines are stably transfected with a nucleic acid molecule encoding the membrane-associated protein, whose expression is driven by an inducible promoter.

Retroviral particles according to the invention may be targeted to a specific cell type by including in the retroviral particles a component, most frequently a polypeptide or carbohydrate, which binds to a cell surface receptor specific for that cell type. Such targeting may be accomplished by preparing a packaging cell line which expresses a chimeric *env* protein comprising a portion of the *env* protein required for viral particle assembly in conjunction with a cell-specific binding domain. In another embodiment, *env* proteins from more than one viral type may be employed, such that resultant viral particles contain more than one species of *env* proteins. (See WO 91/02805 entitled "Recombinant Retroviruses Delivering Vector Constructs to Target Cells" and WO 95/31566 entitled "Compositions and Methods for Targeting Gene Delivery Vehicles", both of which are hereby incorporated by reference.) Yet another embodiment involves inclusion of a cell specific ligand in the retroviral capsid or xenotropic envelope to provide target specificity. In a preferred embodiment at this aspect of the invention, the xenotropic *env* gene employed encodes all or a portion of the xenotropic *env* protein required for retroviral assembly in conjunction with a receptor binding domain of a polypeptide ligand known to interact with a cell surface receptor whose tissue distribution is limited to the cell type(s) to be targeted, *e.g.*, a hematopoietic stem cell. In this regard, it may be preferable to utilize a receptor binding domain which binds receptors expressed at high levels on the target cell's surface.

Non-viral membrane-associated proteins may also be used to enhance targeting of recombinant retroviral particles to hematopoietic stem cells. Representative examples include polypeptides which act as ligands for hematopoietic stem cell surface receptors. Depending on the tissue distribution of the receptor for the protein in question, the recombinant retroviral particle could be targeted to a different subset of hematopoietic stem cells.

When a ligand to be included within the xenotropic envelope is not a naturally occurring membrane-associated protein, it is necessary to associate the ligand with the membrane, preferably by making a "hybrid" or "chimeric" envelope protein. It is important to understand that such hybrid envelope proteins can contain extracellular domains from proteins other than other viral or retroviral *env* proteins. To accomplish this, the gene coding for the ligand can be functionally combined with sequences coding for a membrane-associated domain of the *env* protein. By "naturally occurring membrane associated protein", it is meant those proteins that in their native state exist *in vivo* in association with lipid membrane such as that found associated with a cell membrane or on a viral envelope. As such, hybrid envelopes can be used to tailor the tropism (and effectively increase titers) of a retroviral vector according to the invention, as the extracellular component of *env* proteins

is responsible for specific receptor binding. The cytoplasmic domain of these proteins, on the other hand, play a role in virion formation. The present invention recognizes that numerous hybrid *env* gene products (*i.e.*, specifically, retroviral *env* proteins having cytoplasmic regions and extracellular binding regions which do not naturally occur together) can be generated and may alter host range specificity.

5 In a preferred embodiment, this is accomplished by recombining the gene coding for the ligand (or part thereof conferring receptor binding activity) proximate of the membrane-binding domain of the envelope proteins that stably assemble with a given capsid protein. The resulting construct will code for a bifunctional chimeric protein capable of enhanced cell targeting and inclusion in a retroviral lipid envelope.

10 Vector particles having non-native membrane-associated ligands as described herein, will, advantageously, have a host range determined by the ligand-receptor interaction of the membrane-associated protein. Thus, for targeted delivery to hematopoietic stem cells, a vector particle having altered host range can be produced using the methods of the present invention. The ligand will be selected to provide a host range including hematopoietic stem cells. Many different targeting strategies can be employed in connection with this aspect of the invention. For example, there are a number of progenitor cell types found in bone marrow that differentiate into blood cells. Many blood cells have relatively short life spans and therefore progenitor cells must continually divide and differentiate to replace the lost cells. In a preferred embodiment, gene therapy targets hematopoietic progenitor cells, including pluripotent stem cells. These progenitor cells are known to have unique cellular determinants that permit histological identification, separation from other cell types by various techniques, including fluorescence activated cell sorting (FACS) and positive and negative selection [see U.S. Patent No. 5,061,620], and which can be used as cell receptors for the membrane-associated proteins of the vector particles of the present invention.

25 As used herein, a hematopoietic stem cell is a primitive, or immature, cell capable of self-renewal and which is capable of differentiating into precursor cells of all hematopoietic lineages, *i.e.*, they are said to be "pluripotent." Recombinant vectors according to the invention may be introduced into such cells or any their more differentiated progeny, such as the various primitive progenitors and the more lineage committed precursor cells that give rise to the various hematopoietic cell lineages. One marker for such early hematopoietic cells is CD34, which can be identified using monoclonal antibodies. See U.S. patent 4,714,680; WO 93/25216, published December 23, 1993. WO 93/25216 describes a class of hematopoietic stem cells as having the phenotype CD34<sup>+</sup>/CD38<sup>-</sup>/HLA-DR<sup>-</sup> and lacking the lineage committed antigens CD33, CD10, CD5, and CD71. Representative examples of anti-

CD34 antibodies include 12.8 (Andrews, *et al*, *Blood*, 67:842, 1986) and My10 (Civin, *et al*, *J. Immunol.*, 133:157, 1984, commercially available from Becton Dickinson under the designation HPCA-2). Other antibodies may be also utilized to target a selected cell type, such as anti-CD4 antibodies to target CD4+ T-cells and anti-CD8 antibodies to target CD8+ cells (see generally, Wilchek, *et al*, *Anal. Biochem.*, 171:1, 1988).

The vectors may be constructed to target these cell types for gene delivery by including an expressible gene which encodes a membrane-associated protein that binds to a unique cellular determinant of such hematopoietic progenitor cell types. Examples of such progenitor cell types which could be targeted using recombinant retroviral particles of the present invention include pluripotent stem cells, erythroblasts, lymphoblasts, myeloblasts and megakaryocytes.

Those in the art will also recognize that it is also possible to add ligand molecules exogenously to the retroviral particles which are either incorporated into the lipid envelope or which can be linked chemically to the lipid or protein constituents thereof.

Targeting a retroviral vector carrying a gene of interest to a predetermined locus on a chromosome may also be employed. Clear advantages of such targeting include avoidance of insertional mutagenesis and assuring integration at sites known to be transcriptionally active. Techniques for targeting proviral integration to specific sites include integrase modification. See U.S.S.N. 08/156,789, *supra*.

It is further envisioned that the therapy of the present invention be performed *in vitro*. For *in vitro* therapy (also referred to as "*ex vivo therapy*"), cells are removed and transduced *in vitro*. For recombinant particles having membrane-associated proteins to enhance hematopoietic stem cell targeting, the need to purify the cells to be targeted *in vitro* is optional because the vector would specifically transduce only the targeted cells. Thus, bone marrow samples could be removed from a subject and the desired cell type transduced without the need to perform one or more cell sorting procedures. The transduced cells could then be returned to the same patient or one who is HLA matched.

In addition a wide variety of high affinity binding pairs can be used as targeting elements. Representative examples of include biotin/avidin with an affinity ( $K_D$ ) of  $10^{-15}$  M (Richards, *Meth. Enz.*, 184:3, 1990; Green, *Adv. in Protein Chem.*, 29:85, 1985) and cystatin/papain with an affinity of  $10^{-14}$  M (Bjork, *et al.*, *Biochemistry*, 29:1770, 1990).

A wide variety of other high affinity binding pairs may also be developed, for example, by preparing and selecting antibodies which recognize a selected hematopoietic stem cell antigen with high affinity (see generally, U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993; see also *Monoclonal Antibodies, Hybridomas: A New Dimension*

in *Biological Analyses*, Plenum Press, Kennett, McKearn, and Bechtol, eds., 1980, and *Antibodies: A Laboratory Manual*, Harlow and Lane eds., Cold Spring Harbor Laboratory Press, 1988). The binding pair for such antibodies, typically other antibodies or antibody fragments, may be produced by recombinant techniques (see Huse, *et al.*, *Science*, 246:1275, 1989; see also Sastry, *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:5728, 1989; and Michelle Alting-Mees, *et al.*, *Strategies in Molecular Biology*, 3:1, 1990).

As will be evident to one of ordinary skill in the art given the disclosure provided herein, either member (or molecule) of the affinity binding pair may be coupled to the retroviral particle. Nevertheless, within preferred embodiments of the invention, the larger of the two affinity binding pairs (e.g., avidin of the avidin/biotin pair) is coupled to the retroviral particle. As utilized within the context of targeting, the term "coupled" may refer to either noncovalent or covalent interactions, although generally covalent bonds are preferred. Numerous coupling methods may be utilized, including, for example, use of crosslinking agents such as N-succinimidyl-3-(2-pyridyl dithio) propionate ("SPDP"; Carlson, *et al.*, *J. Biochem.*, 173:723, 1978) and other such compounds known in the art.

In particularly preferred embodiments of the invention, a member of the high affinity binding pair is either expressed on, or included as an integral part of, a retroviral particle, e.g., in the retroviral lipid envelope. For example, a member of the high affinity binding pair may be co-expressed with the envelope protein as a hybrid protein or expressed from an appropriate vector which targets the member of the high affinity binding pair to the cell membrane in the proper orientation.

#### Uses of Recombinant Retroviral Particles

The efficient use of recombinant retroviral particles to infect cells is dependent on the tropism of the envelope protein expressed on the surface of the retroviral particle. The use of a envelope enables infection of cells from different species.

In one aspect, the present invention provides methods for inhibiting the growth of a selected tumor ("cancer") in a human, comprising the step of transducing hematopoietic stem cells *ex vivo* with a vector construct which directs the expression of at least one anti-tumor agent. Within the context of the present invention, "inhibiting the growth of a selected tumor" refers to either (1) the direct inhibition of tumor cell division or metastasis, or (2) immune cell mediated tumor cell lysis, or both, which leads to a suppression in the net expansion of tumor cells. Inhibition of tumor growth by either of these two mechanisms may be readily determined by one of ordinary skill in the art based upon a number of well known



methods, for example, by measuring the tumor size over time, such as by radiologic imaging methods (*e.g.*, single photon and positron emission computerized tomography; *see generally*, "Nuclear Medicine in Clinical Oncology," Winkler, C. (ed.) Springer-Verlag, New York, 1986) or by a variety of imaging agents, including, for example, conventional imaging agents (5 *e.g.*, Gallium-67 citrate) or specialized reagents for metabolite imaging, receptor imaging, or immunologic imaging. In addition, non-radioactive methods such as ultrasound (*see*, "Ultrasonic Differential Diagnosis of Tumors", Kossoff and Fukuda, (eds.), Igaku-Shoin, New York, 1984), may also be utilized to estimate tumor size. Alternatively, for other forms of cancer, inhibition of tumor growth may be determined based upon a change in the  
10 presence of a tumor marker, *e.g.*, prostate specific antigen ("PSA") for the detection of prostate cancer (*see* U.S. Patent No. Re. 33,405), and carcino-embryonic antigen ("CEA") for the detection of colorectal and certain breast cancers. For yet other types of cancers such as leukemia, inhibition of tumor growth may be determined based upon decreased numbers of leukemic cells in a representative blood cell count.

15 Within the context of the present invention, "anti-tumor agent" refers to a compound or molecule which inhibits tumor growth. Representative examples of anti-tumor agents include immune activators and tumor proliferation inhibitors. Briefly, immune activators function by improving immune recognition of tumor-specific antigens such that the immune system becomes "primed." Priming may consist of lymphocyte proliferation, differentiation,  
20 or evolution to higher affinity interactions. The immune system thus primed will more effectively inhibit or kill tumor cells. Immune activation may be subcategorized into immune modulators (molecules which affect the interaction between lymphocyte and tumor cell) and lymphokines, that act to proliferate, activate, or differentiate immune effector cells. Representative examples of immune modulators include CD3, ICAM-1, ICAM-2, LFA-1,  
25 LFA-3,  $\beta$ -2-microglobulin, chaperones, alpha interferon and gamma interferon, B7/BB1 and major histocompatibility complex (MHC). Representative examples of lymphokines include gamma interferon, tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, GM-CSF, CSF-1, and G-CSF. In addition, RNA molecules having intrinsic biological activity may be utilized as anti-tumor agents.

30 Sequences which encode anti-tumor agents may be obtained from a variety of sources. For example, plasmids that contain sequences which encode anti-tumor agents may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, MD), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford, England). Alternatively, known cDNA sequences which encode anti-  
35 tumor agents may be obtained from cells which express or contain the sequences.

Additionally, cDNA or mRNA libraries from specific cell sources can be purchased from commercial sources from which the desired sequences can be readily cloned by conventional techniques, *e.g.*, PCR amplification. Sequences which encode anti-tumor agents may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA  
5 synthesizer model 392, Foster City, CA).

In addition to the anti-tumor agents described above, the present invention also provides anti-tumor agents which comprise a fusion protein of, for example, two or more cytokines, immune modulators, toxins or differentiation factors. Preferred anti-tumor agents in this regard include alpha interferon - Interleukin-2, GM-CSF - IL-4, GM-CSF - IL-2, GM-  
10 CSF - IL-3 (*see* U.S. Patent Nos. 5,082,927 and 5,108,910), GM-CSF - gamma interferon, and gamma interferon - IL-4, with gamma interferon - interleukin-2 being particularly preferred.

Within another embodiments, the anti-tumor agent may further comprise a membrane anchor. The membrane anchor may be selected from a variety of sequences, including, for  
15 example, the transmembrane domain of well known proteins. Generally, membrane anchor sequences are regions of a protein that anchor the protein to a membrane. Customarily, there are two types of anchor sequences that attach a protein to the outer surface of a cell membrane: (1) transmembrane regions that span the lipid bilayer of the cell membrane (proteins containing such regions are referred to integral membrane proteins); and  
20 (2) domains which interact with an integral membrane protein or with the polar surface of the membrane (such proteins are referred to as peripheral, or extrinsic, proteins).

Membrane anchors derived from integral membrane proteins are preferred. Membrane spanning regions typically have a similar structure, with a 20 to 25 amino-acid residue portion consisting almost entirely of hydrophobic residues located inside the  
25 membrane (*see* Eisenberg *et al.*, *Ann. Rev. Biochem.* 53:595-623, 1984). Membrane spanning regions typically have an alpha helical structure (*see* Eisenberg *et al.* at 20; Heijne and Manoil at 109). Within a preferred embodiment, a membrane anchor is fused to the C-terminus of gamma interferon fusion protein, wherein the membrane anchor comprises the gamma-chain of the Fc receptor.

30 Tumorigenicity of an anti-tumor agent can be assessed by various assays. Representative assays include tumor formation in nude mice or rats, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice. In addition to tumorigenicity studies, it is generally preferable to determine the toxicity of an anti-tumor agent. A variety of methods well known to those of skill in the art may be utilized to

measure such toxicity, including for example, clinical chemistry assays which measure the systemic levels of various proteins and enzymes, as well as blood cell volume and number.

Once an anti-tumor agent has been selected, it is placed into a vector construct according to the invention.

5        Such a vector construct can then be packaged into a recombinant retroviral vector and be used to transduce *ex vivo* hematopoietic stem cells which are then re-introduced into the patient. In the context of the present invention, it should be understood that the removed cells may not only be returned to the same patient, but may also be utilized to inhibit the growth of selected tumor cells in another allogeneic human.

#### 10        Preparation and Purification of Recombinant Retroviral Particles

Another aspect of the invention concerns the preparation of recombinant retroviral particles. Recombinant retroviral particles according to the invention can be produced in a variety of ways, as those in the art will appreciate. For example, producer cells, *i.e.*, cells containing all necessary components for retroviral vector packaging (including a nucleic acid molecule encoding the retroviral vector), can be grown in roller bottles, in bioreactors, in hollow fiber apparatus, and in cell hotels. Cells can be maintained either on a solid support in liquid medium, or grown as suspensions. A wide variety of bioreactor configurations and sizes can be used in the practice of the present invention.

20        Cell factories (also termed "cell hotels") typically contain 2, 10, or 40 trays, are molded from virgin polystyrene, treated to provide a Nuclon D™ surface, and assembled by sonic welding one to another. Generally, these factories have two port tubes which allow access to the chambers for adding reagents or removing culture fluid. A 10-layer factory provides 6000 cm<sup>2</sup> of surface area for growing cells, roughly the equivalent of 27 T-225 flasks. Cell factories are available from a variety of manufacturers, including for example Nunc (Baxter, Sanform, ME). Most cell types are capable of producing high titer vector for 3 to 6 days, allowing for multiple harvests. Each cell type is tested to determine the optimal harvest time after seeding and the optimal number of harvest days. Cells are typically initially grown in DMEM supplemented with 2 to 20% FBS in roller bottles until the required number of cells for seeding a cell factory is obtained. Cells are then seeded into the factories and 2 liters (L) of culture supernatant containing vector is harvested later at an appropriate time. Fresh media is used to replenish the cultures.

30        Hollow fiber culture methods may also be used. Briefly, high titer retroviral production using hollow fiber cultures is based on increasing viral concentration as the cells

are being cultured to a high density in a reduced volume of media. Cells are fed nutrients and waste products are diluted using a larger volume of fresh media which circulates through the lumen of numerous capillary fibers. The cells are cultured on the exterior spaces of the capillary fibers in a bioreactor chamber where cell waste products are exchanged for nutrients by diffusion through 30 kD pores in the capillary fibers. Retroviruses which are produced from the cell lines are too large to pass through the pores, and thus concentrate in the hollow fiber bioreactor along side of the cells. The volume of media being cultured on the cell side is approximately 10 to 100 fold lower then volumes required for equivalent cell densities cultured in tissue culture dishes or flasks. This decrease in volume inversely correlates with the fold induction of titer when hollow fiber retroviral titers are compared to tissue culture dishes or flasks. This 10 to 100 fold induction in titer is seen when an individual retroviral producer cell line is amiable to hollow fiber growth conditions. To achieve maximum cell density, the individual cells must be able to grow in very close proximity and on top of each other. Many cell lines will not grow in this fashion and retroviral packaging cell lines based on these types of cell lines may not achieve 10 fold increases in titer. Cell lines which would grow very well would be non-adherent cell line and it is believed that a retroviral producer line based on a non-adherent cell line may reach 100 fold increases in titer compared to tissue culture dishes and flasks.

Regardless of the retroviral particle and production method, high titer (from about  $10^7$ - $10^{11}$  cfu/mL) stocks can be prepared that will cause high level expression of the desired products upon introduction into appropriate cells. When all components required for retroviral particle assembly are present, high-level expression will occur, thereby producing high titer stocks. And while high titer stocks are preferred, retroviral preparations having titers ranging from about  $10^3$  to  $10^6$  cfu/mL may also be employed, although retroviral titers can be increased by various purification methods, as described below.

After production by an appropriate means, the infectious recombinant retroviral particles may be preserved in a crude or purified form. Crude retroviral particles are produced by cultivated infected cells, wherein retroviral particles are released from the cells into the culture media. The virus may be preserved in crude form by first adding a sufficient amount of a formulation buffer to the culture media containing the recombinant virus to form an aqueous suspension.

Recombinant retroviral particles can also be preserved in a purified form. More specifically, prior to the addition of formulation buffer, the crude retroviral preparation described above is clarified by passing it through a filter, and then concentrated, such as by a cross flow concentrating system (Filtron Technology Corp., Nortborough, MA). Within one

embodiment, DNase is added to the concentrate to digest exogenous DNA. The digest is then diafiltrated to remove excess media components and establish the recombinant virus in a more desirable buffered solution. The diafiltrate is then passed over a gel filtration column, such as a Sephadex S-500 gel column, and the purified recombinant virus is eluted.

5        Crude recombinant retroviral preparations can also be purified by ion exchange column chromatography, such as is described in more detail in U.S.S.N. Serial No. 08/093,436. In general, the crude preparation is clarified by passing it through a filter, and the filtrate loaded onto a column containing a highly sulfonated cellulose matrix, wherein the amount of sulfate per gram of cellulose ranges from about 6 - 15  $\mu$ g. The recombinant  
10        retrovirus is eluted from the column in purified form by using a high salt buffer. The high salt buffer is then exchanged for a more desirable buffer by passing the eluate over a molecular exclusion column. The purified preparation may then be formulated or stored, preferably at -70°C.

      Additionally, the preparations containing recombinant retroviruses according to the  
15        invention can be concentrated during purification in order to increase the titer of recombinant retrovirus. A wide variety of methods may be utilized for increasing retroviral concentration, including for example, precipitation of recombinant retroviruses with ammonium sulfate, polyethylene glycol ("PEG") concentration, concentration by centrifugation (either with or without gradients such as PERCOLL™, or "cushions" such as sucrose, use of concentration  
20        filters (e.g., Amicon filtration, Chicago, IL), and 2-phase separations.

      Briefly, to accomplish concentration by precipitation of recombinant retroviruses with ammonium sulfate, ammonium sulfate is added slowly to an appropriate concentration, followed by centrifugation and removal of the ammonium sulfate either by dialysis or by separation on a hydrophobic column.

25        Alternatively, recombinant retroviruses may be concentrated from culture medium with PEG (Green, *et al.*, *PNAS* 67:385, 1970; Syrewicz, *et al.*, *Appl. Micro.* 24:488, 1972). Such methods are rapid, simple, and inexpensive. However, like ammonium sulfate precipitation, use of PEG also concentrates other proteins from solution.

      Within other embodiments, recombinant retroviruses may be concentrated by  
30        centrifugation, and more particularly, low speed centrifugation, which avoids difficulties associated with pelleting that accompanies high speed centrifugation (e.g., virus destruction or inactivation).

      Recombinant retroviruses according to the invention may also be concentrated by an aqueous two-phase separation method. Briefly, polymeric aqueous two-phase systems may  
35        be prepared by dissolving two different non-compatible polymers in water. Many pairs of

water-soluble polymers may be utilized in the construction of such two-phase systems, including for example PEG or methylcellulose, and dextran or dextran sulfate (*see* Walter and Johansson, *Anal. Biochem.* 155:215, 1986; Albertsson, "Partition of Cell Particles and Macromolecules" Wiley, New York, 1960). As described in more detail below in Example 5 7, utilizing PEG at concentrations ranging from 5% to 8% (preferably 6.5%), and dextran sulfate at concentrations ranging from 0.4% to 1% (preferably 0.4%), an aqueous two-phase system may be established suitable for purifying recombinant retroviruses. Utilizing such procedures, approximate 100-fold concentration can be achieved with yields of approximately 50% or more of the total starting retrovirus.

10 For purposes of illustration, a representative concentration process which combines several concentration steps is set forth below. Briefly, recombinant retroviruses may be prepared either from roller bottles, cell factories, or bioreactors prior to concentration. Removed media containing the recombinant retrovirus may be frozen at -70°C, or more preferably, stored at 2°C to 8°C in large pooled batches prior to processing.

15 For material obtained from a bioreactor, the recombinant retrovirus pool is first clarified through a 0.8 µm filter (1.2 µm glass fiber pre-filter, 0.8 µm cellulose acetate) connected in series with a 0.65 µm filter. This filter arrangement provides approximately 2 square feet (sq. ft.) of filter, and allows processing of about 15-20 L of pooled material before clogging. For material obtained from roller bottles or cell factories, a single 0.65 µm 20 cartridge (2 sq. ft.) normally suffices for volumes up to 40 L. For 80 L cell factory processes, a 5 sq. ft. filter may be required.

Preferably, after clarification the filter is rinsed with buffer (*e.g.*, 150 mM NaCl, 25 mM Tris, pH 7.2-7.5). Following clarification, recombinant retroviruses are concentrated by tangential flow ultrafiltration utilizing cassettes with a 300,000 mw cut off. For bioreactor 25 material (containing 12% to 16% FBS), 4 to 5 L of material may be concentrated per cassette. For roller bottles or cell factories at 12 to 16% FBS, 5 to 6 L of material may be concentrated per cassette. Finally, for cell factories containing 10% FBS, 8 to 9 L of material may be concentrated per cassette. Utilizing such procedures at an appropriate pressure differential between filtrate and retentate, up to 80 L of material may be 30 concentrated to a volume of less than 500 mL in under two hours. This process also provides a yield of about 80%.

Following the ultrafiltration step, DNase may be added to a concentration of 50 U/mL, and recirculated at a lower pump speed with the filtrate line closed for 30 minutes. Discontinuous diafiltration is then accomplished by adding additional buffer and utilizing the

same cross differential pressure as before. Generally, recovery after this step is approximately 70%.

Concentrated material is then subjected to column chromatography on a Pharmacia S-500 HG size exclusion gel, utilizing 50 mM NaCl and 25 mM Tris pH 7.2-7.5 as minimum salt and ionic strength concentrations. Generally, recombinant retroviruses elute off in the first peak.

Tangential flow filtration may once again be utilized to further reduce the volume of the preparation, after which the concentrated material is sterilized by filtration through a 0.2  $\mu$ m Millipore filter (Philadelphia, PA).

As an alternative to *in vivo* production, the retroviral packaging proteins may be produced, together or separately, from appropriate cells. However, instead of introducing a nucleic acid molecule enabling production of the viral vector, an *in vitro* packaging reaction is conducted comprising the *gag*, *pol*, and *xen* proteins, the retroviral vector, tRNA, and other necessary components. The resulting retroviral particles can then be purified and, if desired, concentrated.

#### Formulation Of Pharmaceutical Compositions

Another aspect of the invention relates to pharmaceutical compositions comprising recombinant retroviral vectors as described above, in combination with a pharmaceutically acceptable carrier or diluent, while another aspect is directed toward a method for preserving an infectious recombinant retrovirus for subsequent reconstitution such that the recombinant retrovirus is capable of infecting mammalian cells upon reconstitution. The methods described can be used to preserve a variety of different viruses, including recombinant type C retroviruses such as gibbon ape leukemia virus, feline leukemia virus and xeno-, poly- and amphotropic murine leukemia virus (Weiss, *et al.*, *RNA Tumor Viruses*, 2d ed. 1985). See U.S.S.N. 08/153,342.

Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions, preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin (HSA). A particularly preferred composition comprises a recombinant retrovirus in 10 mg/mL mannitol, 1 mg/mL HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl. In this case, since the recombinant retroviral particle represents approximately 1  $\mu$ g of material, it may be

less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months.

Pharmaceutical compositions of the present invention may also additionally include factors which stimulate hematopoietic stem cell division, and hence, uptake and  
5 incorporation of vector constructs according to the invention.

Particularly preferred methods and compositions for preserving recombinant retroviruses are described in U.S.S.N. 08/135,938, filed October 12, 1993, and U.S. Serial No. 8/153,342, filed November 15, 1993.

The use of recombinant retroviruses to transduce hematopoietic stem cells useful in  
10 treating patients requires that the product be able to be transported and stored for long periods at a desired temperature such that infectivity and viability of the recombinant retrovirus is retained. The difficulty of preserving recombinant retroviruses absent low temperature storage and transport presents problems in Third World countries, where adequate refrigeration capabilities are often lacking.

15 The initial stabilization of materials in dry form to the preservation of antitoxins, antigens and bacteria has been described (Flosodort, *et al.*, *J. Immunol.*, 29:389, 1935). However, a limitation in this process included partial denaturation of proteins when dried from an aqueous state at ambient temperatures. Drying from the frozen state helped reduce this denaturation and led to efficient preservation of other biological materials, including  
20 bacteria and viruses (Stamp, *et al.*, *J. Gen. Microbiol.*, 1:251, 1947; Rowe, *et al.*, *Virology*, 42:136, 1970; and Rowe, *et al.*, *Cryobiology*, 8:153, 1971). More recently, sugars such as sucrose, raffinose, glucose and trehalose were added in various combinations as stabilizing agents prior to lyophilization of viruses. The use of sugars enhanced recovery of viable viruses, for research purposes which require that only some virus survive for later  
25 propagation.

Recombinant retroviruses according to the invention can be stored in liquid, or preferably, lyophilized form. Factors influencing stability include the formulation (liquid, freeze dried, constituents thereof, *etc.*) and storage conditions, including temperature, storage container, exposure to light, *etc.* Alternatively, retroviral particles according to the  
30 invention can be stored as liquids at low temperatures. In a preferred embodiment, the recombinant retroviruses of the invention are formulated to preserve infectivity in a lyophilized form at elevated temperatures, and for this form to be suitable for injection into patients following reconstitution.

Recombinant retroviral particles comprising retroviral vector constructs according to  
35 the invention can be formulated in crude or, preferably, purified form. Crude retroviral



preparations may be produced by various cell culture methods, where retroviral particles are released from the cells into the culture media. Recombinant retroviral particles may be preserved in crude form by adding a sufficient amount of formulation buffer. Typically, the formulation buffer is an aqueous solution containing various components, such as one or  
5 more saccharides, high molecular weight structural additives, buffering components, and/or amino acids.

The recombinant retroviruses described herein can also be preserved in a purified form. For instance, prior to the addition of formulation buffer, crude preparations as described above may be clarified by filtration, and then concentrated, such as by a cross flow  
10 concentrating system (Filtron Technology Corp., Nortborough, MA). DNase may be added to the concentrate to digest exogenous DNA, followed by diafiltration to remove excess media components and substitute in a more desirable buffered solution. The diafiltrate may then be passed over a gel filtration column, such as a Sephadex™ S-500 gel column, and the eluted retroviral particles retained. A sufficient amount of formulation buffer may then be  
15 added to the eluate to reach a desired final concentration of the constituents and to minimally dilute the retroviral preparation. The aqueous suspension can then be stored, preferably at -70°C, or immediately formulated.

In an alternative procedure, the crude preparation can be purified by ion exchange column chromatography, as described in co-owned U.S.S.N. 08/093,436, filed July 16, 1993. Briefly, the crude recombinant retrovirus is clarified by filtration and then loaded onto a  
20 column comprising a highly sulfonated cellulose matrix. Highly purified recombinant retrovirus is eluted from the column using a high salt buffer, which is then exchanged for a more desirable buffer by passing the eluate over a molecular exclusion column. After recovery, formulation buffer may then be added to adjust the final concentration, as discussed  
25 above, followed by low temperature storage, preferably at -70°C, or immediate formulation.

When a dried formulation is desired, an aqueous preparation containing a crude or purified retroviral preparation can be prepared by lyophilization or evaporation. Lyophilization involves cooling the aqueous preparation below the glass transition  
30 temperature or below the eutectic point temperature of the solution, and removing water by sublimation. For example, a multistep freeze drying procedure as described by Phillips *et al.* (*Cryobiology*, 18:414, 1981) can be used to lyophilize the formulated recombinant virus, preferably from a temperature of -40°C to -45°C. The resulting composition should contain less than 10% water by weight. Once lyophilized, such a preparation is stable and may be stored at -20°C to 25°C.

In an evaporative method, water is removed by evaporation from the retroviral preparation aqueous suspension at ambient temperature. Evaporation can be accomplished by various techniques, including spray drying (*see* EP 520,748), where the preparation is delivered into a flow of preheated gas, usually air, whereupon water rapidly evaporates from droplets of the suspension. Spray drying apparatus are available from a number of manufacturers (*e.g.*, Drytec, Ltd., Tonbridge, England; Lab-Plant, Ltd., Huddersfield, England). Once dehydrated, the recombinant retroviral preparation is stable and may be stored at -20°C to 25°C. The resulting moisture content of the dried or lyophilized preparation may be determined through use of a Karl-Fischer apparatus (EM Science Aquastar<sup>®</sup> V1B volumetric titrator, Cherry Hill, NJ), or through a gravimetric method. Once dehydrated, the recombinant retrovirus is stable and may be stored at -20°C to 25°C.

As mentioned previously, aqueous preparations comprising retroviruses according to the invention used for formulation are typically composed of one or more saccharides, high molecular weight structural additives, buffering components, and water, and may also include one or more amino acids. It has been found that the combination of these components acts to preserve the activity of the recombinant retrovirus upon freezing and lyophilization, or drying through evaporation. *See* co-owned U.S.S.N. 08/153,342, filed November 15, 1993. Various saccharides may be used alone or in combination, including sucrose, mannitol, glucose, trehalose, inositol, fructose, maltose, and galactose, with lactose being particularly preferred. The concentration of the saccharide can range from 0.1% to 30% by weight, preferably from about 1% to 12% by weight. A particularly preferred concentration of lactose is 3% to 4% by weight. Additionally, saccharide combinations can also be employed, including lactose and mannitol or sucrose and mannitol. It will also be evident to those skilled in the art that it may be preferable to use certain saccharides in the aqueous solution when the lyophilized formulation is intended for room temperature storage. Specifically, disaccharides, such as lactose or trehalose, are preferred for such formulations.

One or more high molecular weight structural additives may be used to aid in preventing retroviral aggregation during freezing and provides structural support in the lyophilized or dried state. In the context of the present invention, structural additives are considered to be of "high molecular weight" if they are greater than 5000 daltons. A preferred high molecular weight structural additive is human serum albumin (HSA), although other substances may also be used, such as hydroxyethyl-cellulose, hydroxymethyl-cellulose, dextran, cellulose, gelatin, povidone, *etc.* Preferably, the concentration of the high molecular weight structural additive can range from 0.05% to 20%, with 0.1% to 10% by weight being preferred, and a concentration of 0.1% by weight HSA being particularly preferred.

Amino acids, if present, tend to further preserve retroviral infectivity. In addition, amino acids function to further preserve retroviral infectivity during sublimation of the cooled aqueous suspension and while in the lyophilized state. A preferred amino acid is arginine, but other amino acids such as lysine, ornithine, serine, glycine, glutamine, asparagine, glutamic acid or aspartic acid can also be used. Preferably, the amino acid concentration ranges from 0.1% to 10% by weight. A particularly preferred arginine concentration is 0.1% by weight.

A variety of buffering components may be used to maintain a relatively constant pH, depending on the pH range desired, preferably between 7.0 and 7.8. Suitable buffers include phosphate buffer and citrate buffer. A particularly preferred formulation pH is 7.4, and a preferred buffer is tromethamine.

It may also be preferable to include in the formulation a neutral salt to adjust the final iso-osmotic salt concentration. Suitable neutral salts include sodium chloride, potassium chloride, and magnesium chloride, with sodium chloride being preferred.

A particularly preferred method of preserving recombinant retroviruses in a lyophilized state for subsequent reconstitution comprises: (a) preparing an aqueous recombinant retroviral preparation comprising, in addition to the recombinant retrovirus, about (i) 4% by weight of lactose, (ii) 0.1% by weight of HSA, (iii) 0.03% or less by weight of NaCl, (iv) 0.1% by weight of arginine, and a sufficient amount of tromethamine to provide a pH of approximately 7.4; (b) cooling the preparation to a temperature of about -40°C to -45°C to form a frozen preparation; and (c) removing water from the frozen preparation by sublimation to form a lyophilized composition having less than 2% water by weight. It is preferred that the recombinant retrovirus be replication defective and suitable for administration into humans cells upon reconstitution.

The lyophilized or dehydrated retroviruses of the subject invention may be reconstituted using a variety of substances, but are preferably reconstituted using water. In certain instances, dilute salt solutions which bring the final formulation to isotonicity may also be used. In addition, it may be advantageous to use aqueous solutions containing components known to enhance the activity of the reconstituted virus. Such components include cytokines, such as IL-2, polycations, such as protamine sulfate, or other components which enhance the transduction efficiency of the reconstituted virus. Lyophilized or dehydrated recombinant virus may be reconstituted with any convenient volume of water or the reconstituting agents noted above that allow substantial, and preferably total solubilization of the lyophilized or dehydrated sample.

Administration of Recombinant Retroviral Particles

In another aspect of the present invention, methods are provided for treating human patients afflicted with a variety of diseases, including a genetic disease, cancer, an infectious disease, an autoimmune disease, and inflammatory disease, a cardiovascular disease, and a degenerative disease. Examples of genetic diseases include but are not limited to; thalassemia, phenylketonuria, Lesch-Nyan syndrome, SCID, hemophilia A and B, cystic fibrosis, Duchenne's muscular dystrophy, inherited emphysema, familial hypercholesterolemia, and Gaucher's disease. Examples of cancers include but are not limited to; solid tumors, leukemias and lymphomas. Representative examples include melanomas, colorectal carcinomas, lung carcinomas (including large cell, small cell, squamous and adeno-carcinomas), renal cell carcinomas, cervical cancer, adult T-cell lymphoma leukemia, and breast adeno-carcinomas. Infectious diseases include but not limited to; hepatitis, tuberculosis, malaria, human immunodeficiency virus, herpes virus, tetanus, dysentery, shigella, FeLV, and FIV. Degenerative diseases include but are not limited to; Alzheimer's disease, multiple sclerosis, muscular dystrophy, amyotrophic lateral sclerosis. Inflammatory diseases include rheumatoid arthritis, spinal meningitis, and pancreatitis. Autoimmune diseases include diabetes, uveitis, HIV, and SCID. Cardiovascular diseases include, chronic rheumatic heart disease, arteriosclerosis, mitral valve and aortic stenosis, myocarditis, pericarditis, Marfan's syndrome, Ehlers-Danlos syndrome, Churg-Strauss syndrome, and scleroderma.

Each of these methods comprise administering to a human a recombinant retroviral particle preparation as described above, such that a therapeutically efficacious amount of gene product encoded by the gene of interest carried on the vector construct is produced. As used herein, a "therapeutically effective amount" of a gene product expressed from a vector construct according to the invention is an amount that achieves a desired therapeutic benefit in a patient to an extent greater than that observed when the patient was not treated with the gene product. For instance, when the gene product is factor VIII, a "therapeutically effective amount" refers to the amount of factor VIII needed to produce therapeutically beneficial clotting and will thus generally be determined by each patient's attending physician, although serum levels of about 0.2 ng/mL (about 0.1% of "normal" levels) or more will be therapeutically beneficial. When the gene product is an RNA molecule with intrinsic biological activity, such an antisense RNA or ribozyme, a "therapeutically effective amount" is an amount sufficient to achieve a clinically relevant change in the patient's condition through reduced expression of the harmful gene product, most often a protein. In a

preferred embodiment, the RNA molecule with intrinsic biological activity will be expressed in transduced hematopoietic stem cells in molar excess to the targeted RNA molecule. Expression levels of the heterologous and targeted RNAs can be determined by various assays, *e.g.*, by PCR analysis.

5 Typically, the dosage for *ex vivo* gene modified hematopoietic stem cells will be in the range of  $10^5$  to  $10^8$  cells per kilogram patient body weight depending upon the purity of stem cells in the starting population. Thus, for example, for CD34<sup>+</sup> selected cells, usually  $10^7$  to  $10^8$  cells will be transduced *ex vivo* for re-infusion into a patient; for more highly enriched stem cell populations, such as CD34<sup>+</sup> Thy-1<sup>-</sup>Lin<sup>-</sup> selected cells, usually from  
10  $10^5$  to  $10^7$  cells will be transduced and re-infused. The stem cell population will usually be transduced at multiplicity of infection (MOI) of 10 to 1000, typically around 100 infectious recombinant retroviral particles per cell.

Preferably, high titer preparations of retroviral vectors are used for *ex vivo* transduction. The volume that the high titer preparation is delivered in *ex vivo* is preferably  
15 not greater than 10% of the culture medium volume of the cell culture. More preferably, the volume of the high titer preparation is less than 1%, still more preferably less than 0.1%, and still more preferably less than 0.001% of the total cell culture volume. Additionally the retrovirus is delivered in a medium that is free of agents that disturb or are toxic to the transduced cells in culture (eg. in an aqueous liquid with a composition similar to that of cell  
20 culture medium).

#### Hematopoietic Stem Cells

A pluripotent hematopoietic stem cell may be defined as follows: (1) a cell which  
25 gives rise to progeny in all defined hematolymphoid lineages; and (2) a cell which is capable of fully reconstituting a seriously immunocompromised host of all blood cell types and their progenitors, including the pluripotent hematopoietic stem cell by self renewal.

"Hematopoietic stem cells" refers to a population of hematopoietic cells having all of the long-term engrafting potential *in vivo*. Animal models for long-term engrafting potential  
30 of candidate human hematopoietic stem cell populations include the SCID-hu bone model (Kyoizumi et al. (1992) Blood 79:1704; Murray et al. (1995) Blood 85(2) 368-378) and the in utero sheep model (Zanjani et al. (1992) J. Clin. Invest. 89:1179. For a review of animal models of human hematopoiesis, see Srour et al. (1992) J. Hematother. 1:143-153 and the references cited therein. At present, the best in vitro assay for stem cells is the long-term  
35 culture-initiating cell (LTCIC) assay, based on a limiting dilution analysis of the number of

clonogenic cells produced in a stromal co-culture after 5-8 weeks. Sutherland et al. (1990) Proc. Natl Acad. Sci. 87:3584-3588. The LTCIC assay has been shown to correlate with another commonly used stem cell assay, the cobblestone area forming cell (CAFC) assay, and with long-term engrafting potential in vivo. Breems *et al.* (1994) Leukemia 8:1095.

5 For use in the present invention, a highly enriched stem cells population is preferred in order to maximize efficiency of gene transfer into the desired target cells. As described more fully below, an enriched stem cell population is exemplified by a population of cells selected by expression of the CD34 marker. In LTCIC assays, a population enriched in CD34+ cells will have an LTCIC frequency in the range of 1/50 to 1/500, more usually in the  
10 range of 1/50 to 1/200. Preferably, the stem cell population will be more highly enriched for stem cells than that provided by a population selected on the basis of CD34+ expression alone. By use of various techniques described more fully below, a highly enriched stem cell population may be obtained. A highly enriched stem cell population will typically have an LTCIC frequency in the range of 1/5 to 1/100, more usually in the range of 1/10 to 1/50.  
15 Preferably it will have an LTCIC frequency of at least 1/50. Exemplary of a highly enriched stem cell population is a population having the CD34+Thy1<sup>+</sup>Lin<sup>-</sup> phenotype as described in U.S. Patent No. 5,061,620. A population of this phenotype will typically have an average LTCIC frequency of approximately 1/20. Murray et al. (1995); Lansdorp et al. (1993) J. Exp. Med. 177:1331.

20 Hematopoietic stem cells may be isolated from any known source of stem cells, including bone marrow, mobilized peripheral blood (MPB), and umbilical cord blood. Initially, bone marrow cells may be obtained from a source of bone marrow, including ileum (*e.g.*, from the hip bone via the iliac crest), tibia, femur, spine, or other bone cavities. Other sources of hematopoietic stem cells include embryonic yolk sac, fetal liver, and fetal spleen.

25 For isolation of bone marrow, an appropriate solution may be used to flush the bone, including saline solution, conveniently supplemented with fetal calf serum (FCS) or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from about 5 to 25 mM. Convenient buffers include HEPES, phosphate buffers and lactate buffers. Otherwise bone marrow may be aspirated from the bone in accordance  
30 with conventional techniques.

Methods for mobilizing hematopoietic stem cells into the peripheral blood are known in the art and generally involve treatment with chemotherapeutic drugs (*e.g.*, cyclophosphamide or etoposide), cytokines (*e.g.*, GM-CSF, G-CSF or IL-3), or combinations thereof. Typically, apheresis for total white cells begins when the total white  
35 cell count reaches 500-2000 cells/ml and the platelet count reaches 50,000/ml. In order to

maximize hematopoietic stem cell recovery from MPB, daily apheresis samples may be monitored for cells expressing CD34 and/or Thy-1. Desirably, the collection of peripheral blood leukocytes begins when an increase in CD34<sup>+</sup> and/or Thy-1<sup>+</sup> cells is detected in order to obtain a sample during the peak of stem cell mobilization. While CD34 expression  
5 normally correlates with stem cell mobilization, in some cases it does not. Therefore, it is preferable to use the Thy-1 marker alone or in combination with ~~CD34~~ to monitor stem cell mobilization (Murray, *et al.* 1995).

Various techniques may be employed to separate the cells by initially removing cells of dedicated lineage ("lineage-committed" cells). Monoclonal antibodies and monoclonal  
10 antibody fragments are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation. The antibodies (or antibody fragments) may be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the viability of the fraction to be collected.

The use of separation techniques include those based on differences in physical  
15 (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rhodamine 123 and DNA-binding dye Hoechst 33342). Procedures for separation may include magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody,  
20 including complement and cytotoxins, and "panning" with antibody attached to a solid matrix or any other convenient technique. Techniques providing accurate separation include flow cytometry which can have varying degrees of sophistication, *e.g.*, a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, *etc.*

A large proportion of the differentiated cells may be removed by initially using a  
25 relatively crude separation, where major cell population lineages of the hematopoietic system, such as lymphocytic and myelomonocytic, are removed, as well as minor populations, such as megakaryocytic, mast cells, eosinophils and basophils. Usually, at least about 70 to 90 percent of the hematopoietic cells will be removed.

"Positive selection" refers to a selection procedure whereby the cell population of  
30 interest expresses the marker used as the basis for selection and, therefore, cells expressing the marker are retained and those not expressing the marker are discarded. "Negative selection" refers to a selection procedure whereby the cell population of interest does not express the marker or have the characteristic that is used as the basis for selection and, therefore, cells having the specified marker or characteristic are discarded and those not  
35 having the specified marker or characteristic are retained. Thus, positive selection

techniques may be employed to select stem cells based on, *e.g.*, CD34 expression. One technique providing positive selection for CD34<sup>+</sup> cells with high purity is described in PCT patent application No. WO94/02016, in which CD34<sup>+</sup> cells are selected using a hapten conjugated anti-CD34 antibody and a hapten competition release system.

5 Concomitantly or subsequent to a gross separation providing for positive selection, *e.g.*, using the CD34 marker, a negative selection may be carried out, where antibodies to lineage-specific markers present on dedicated cells are employed and the lineage-committed removed, *e.g.*, by magnetic bead depletion or flow cytometer. For the most part, these markers include CD2<sup>-</sup>, CD3<sup>-</sup>, CD7<sup>-</sup>, CD8<sup>-</sup>, CD10<sup>-</sup>, CD14<sup>-</sup>, CD15<sup>-</sup>, CD16<sup>-</sup>, CD19<sup>-</sup>, CD20<sup>-</sup>,  
10 CD33<sup>-</sup> and glycophorin A<sup>-</sup>. Normally, negative selection will yield a cell population which is at least CD14<sup>+</sup> and CD15<sup>+</sup>, and preferably which is at least CD2<sup>+</sup>, CD14<sup>+</sup>, CD15<sup>+</sup>, CD16<sup>+</sup>, CD19<sup>+</sup> and glycophorin A<sup>+</sup>. As used herein, Lin<sup>-</sup> refers to a cell population lacking at least one lineage-specific marker. The hematopoietic cell composition may then be further separated using positive selection for the Thy-1 marker and/or selection for rhodamine<sup>10</sup>,  
15 whereby a highly enriched hematopoietic stem cell population is achieved. See Table 1.

The purified hematopoietic stem cells have low side scatter and low to medium forward scatter profiles by FACS analysis. Cytospin preparations show the enriched stem cells to have a size between mature lymphoid cells and mature granulocytes. Cells may be selected based on light-scatter properties as well as their expression of various cell surface  
20 antigens.

While it is believed that the particular order of separation is not critical to this invention, the order indicated is preferred. Preferably, cells are initially separated by a crude separation, followed by a fine separation, with positive selection of one or more markers associated with stem cells and negative selection for one or more markers associated with  
25 lineage committed cells. Compositions highly enriched in hematopoietic stem cells may be achieved in this manner. The desired hematopoietic stem cells are exemplified by a population with the CD34<sup>+</sup>Thy-1<sup>+</sup>Lin<sup>-</sup> phenotype.

A hematopoietic stem cell composition is characterized by being able to be maintained in culture for extended periods of time, being capable of selection and transfer to  
30 secondary and higher order cultures, and being capable of differentiating into the various lymphocytic and myelomonocytic lineages, particularly B and T lymphocytes, monocytes, macrophages, neutrophils, erythrocytes and the like.

The stem cells may be grown in culture in an appropriate nutrient medium, including conditioned medium, a co-culture with an appropriate stromal cell line or a medium  
35 comprising a combination of growth factors sufficient to maintain the growth of



hematopoietic cells. For conditioned media or co-cultures, various stromal cell lines may be used. Since human stromal cell lines are not required, other stromal cell lines may be employed, including rodentiae, particularly murine stromal cell lines. Suitable murine stromal cell lines include AC3 and AC6, which are described in Whitlock, *et al. Cell* 48:1009, 1987.

- 5 Preferably, the stromal cell line used is a passage of AC6, AC6.21 (otherwise referred to as SyS1).

Cytokines may also be added, including, *e.g.*, leukemia inhibitory factor (LIF), interleukins, colony stimulating factors, and stem cell factor (SCF, also known as steel factor, c-kit ligand, MGF). Of particular interest are LIF, stem cell factor, IL-3, IL-6, GM-CSF, G-CSF, MIP-1 $\alpha$ , the flk2/flt3 ligand, and TPO/npl ligand. The factors which are employed may be naturally occurring or synthetic, *e.g.*, prepared recombinantly, and preferably are human. The amount of the factors used will generally be in the range of about 1 ng/ml to 100 ng/ml. Generally, for LIF, the concentration will be in the range of about 1 ng/ml to 100 ng/ml, more usually 5 ng/ml to 30 ng/ml; for IL-3, the concentration will be in the range of about 5 ng/ml to 100 ng/ml, more usually 5 ng/ml to 50 ng/ml; for IL-6, the concentration will be in the range of about 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 20 ng/ml, and for GM-CSF, the concentration will generally be 5 ng/ml to 50 ng/ml, more usually 5 ng/ml to 20 ng/ml, and for SCF the concentration will generally be 10 ng/ml to 150 ng/ml, and usually 50 ng/ml to 100 ng/ml.

- 20 In one embodiment, the hematopoietic stem cells are optionally expanded prior to or after retroviral transduction. During expansion, the growth factors may be present only during the initial course of the stem cell growth and expansion, usually at least 24 hours, more usually at least about 48 hours to 4 days, or may be maintained during the course of the expansion.

- 25 For use in clinical settings, it is preferable to transduce the hematopoietic stem cells without prior or subsequent expansion. In one embodiment therefore, the stem cells are cultured with or without cytokines in an appropriate medium, transduced with the appropriate vector, cultured for approximately 72 hours and reintroduced into the host. In one embodiment of the invention, at least about 0.1% of the hematopoietic stem cells in a given hematopoietic cell population are transduced with a recombinant retroviral particle according to the invention. In another embodiment, at least about 1% of the hematopoietic stem cells in a given hematopoietic cell population are transduced with a recombinant retroviral particle according to the invention, while in yet another embodiment of the invention, at least about 5% of the hematopoietic stem cells in a given hematopoietic cell population are transduced with a recombinant retroviral particle according to the invention.
- 35

In a preferred embodiment of the invention, at least about 10% of the hematopoietic stem cells in a given hematopoietic cell population are transduced with a recombinant retroviral particle as described herein. In another preferred embodiment, at least about 25% of the hematopoietic stem cells in a given hematopoietic cell population are transduced. In particularly preferred embodiments, at least about 50% of the hematopoietic stem cells in a given hematopoietic cell population are transduced. Even more preferred are embodiments where at least about 60%, 70%, 75%, 80%, 90%, or 95% of the hematopoietic stem cells in a given hematopoietic cell population are transduced with a recombinant retroviral particle as described herein.

Transduction may be accomplished by the direct co-culture of stem cells with recombinant retroviral particle producer cells, *e.g.*, by the method of Bregni, *et al.*, *Blood* 80:1418, 1992. For clinical applications, however, transduction by culturing the hematopoietic stem cells with recombinant retroviral supernatant alone or with purified retroviral preparations as described herein, in the absence of stromal cells, is preferred. Transductions may be performed by culturing the hematopoietic stem cells with the virus for from about four hours to six days. Preferably, transduction is carried out for three days, with the media replaced daily with media containing fresh recombinant retrovirus particles. Alternatively, the stem cells may be cultured in the presence of the retrovirus for several hours, *e.g.*, four hours, daily for three to four days, with fresh media replacing the virus-containing media each day. In addition, the cell/virus preparations may be centrifuged. Typically, growth factors will be included in such amounts and concentration to maintain cell viability and induce cell cycling. Normally the cultures will include at least stem cell factor (SCF, also known as steel factor, MGF, and c-kit ligand), IL-3, and IL-6. Other cytokines of interest include leukemia inhibitory factor (LIF), G-CSF, GM-CSF, MIP-1, flk2/flt3 ligand, and TPO. Polycations, such as protamine sulfate, polybrene and the like, will generally be included to promote binding. Protamine sulfate and polybrene are typically used preferably at a concentration of 4 ng/ml).

Gene transfer into hematopoietic stem cells may be used to treat a variety of neoplastic, infectious or genetic diseases. For example, one may introduce genes that confer resistance to chemotherapeutic agents, thereby protecting the progeny hematopoietic cells, allowing higher doses of chemotherapy and thereby improving the therapeutic benefit. For instance, the *mdr1* gene (see U.S. Patent No. 5,206,352) may be introduced into hematopoietic stem cells to provide increased resistance to a wide variety of chemotherapeutic drugs which are exported by the *mdr1* gene product, in combination with the administration of chemotherapeutics such as taxol, *e.g.*, for breast cancer treatment.

Similarly, genes that provide increased resistance to alkylating agents, such as melphalan, may be introduced into hematopoietic stem cells in conjunction with high dose chemotherapy.

5 For viral infections that primarily affect hematolymphoid cells, stem cells may be modified to endow the progeny with resistance to the infectious agent. In the case of human immunodeficiency virus (HIV), for example, specific, sense, antisense or ribozyme sequences may be introduced that interfere with viral infection or replication in the target cells. Alternatively, the introduced gene products may serve as "decoys" by binding essential viral proteins, thereby interfering with the normal viral life cycle and inhibiting replication.

10 Alternatively, hematopoietic stem cells may be modified to produce a product to correct a genetic deficiency, or where the host has acquired a genetic deficiency through a subsequent disease. Genes that may correct a genetic deficiency include adenosine deaminase for the treatment of ADA<sup>-</sup> severe combined immunodeficiency; glucocerebrosidase for the treatment of Gaucher's disease; beta-globin for the treatment of sickle cell anemia; and factor VIII or factor IX for the treatment of hemophilia.

15 In many situations, cell immunotherapy involves removal of bone marrow or other source of hematopoietic stem cells from a human host, isolating the stem cells from the source and optionally expanding the stem cells. Meanwhile, the host may be treated to partially, substantially or completely ablate native hematopoietic capability. The isolated hematopoietic stem cells may be modified prior to or during this period of time, so as to provide hematopoietic stem cells having the desired genetic modification. After completion of the treatment of the host, the modified hematopoietic stem cells are reintroduced to the host to provide for expression of the foreign gene(s), and to reconstitute a functional hematopoietic system, if necessary. The methods of stem cell removal, host ablation and hematopoietic stem cell repopulation are known in the art. If necessary, the process may be repeated to ensure substantial repopulation of the modified stem cells.

20 To ensure that the hematopoietic stem cells have been successfully modified, a vector-specific probe, or PCR using vector-specific primers, may be used to verify the presence of the vector construct in the transduced stem cells or their progeny. In addition, the cells may be grown under various conditions to ensure that they are capable of maturation to all of the hematopoietic lineages while maintaining the capability, as appropriate, of the introduced DNA. Various tests *in vitro* and *in vivo* may be employed to ensure that the pluripotent capability of the stem cells has been maintained.

30 The compositions comprising hematopoietic stem cells provide for production of myeloid cells and lymphoid cells in appropriate cultures. In each of the cultures, mouse or

human stromal cells are provided, which may come from various sources, including, but not limited to, AC3, AC6 or stromal cells derived from mouse or human bone marrow by selection for the ability to maintain hematopoietic stem cells, and the like. Preferably, the stromal cells are AC6.21 and the ability to produce B lymphocytes and myeloid cells is  
5 determined in cultures supplied with LIF and IL-6. Generally, after 3 to 6 weeks of culture ~~on~~ AC6.21 stromal cells, the cells are analyzed by FACS for expression of CD19 (a B cell marker) and CD33 (a myeloid cell marker). Additionally, the hematopoietic stem cells can be analyzed for the ability to give rise to B cells, T cells and myelomonocytic cells in *in vivo* assays, as described below.

10 To demonstrate differentiation to T cells, fetal thymus is isolated and cultured from 4 to 7 days at 25°C, so as to deplete substantially the lymphoid population. The cells to be tested for T-cell activity are then microinjected into the thymus tissue, where the HLA of the population which is injected is mismatched with the HLA of the thymus cells. The thymus tissue may then be transplanted into a scid/scid mouse as described in US Patent No.  
15 5,147,784, particularly transplanting under the kidney capsule. Mice are sacrificed 6 to 7 weeks after transplantation and the thymus graft recovered and reduced to a single cell suspension. Donor-derived cells are detected by HLA staining and thymus differentiation analyzed by CD4 and CD8 staining and FACS analysis.

Further demonstration of the sustained regenerative ability of hematopoietic stem cell  
20 populations may be accomplished by the detection of continued myeloid and B-lymphoid cell production in the SCID-hu bone model. See Kyoizumi, *et al.*, *Blood* 79:1704, 1992. Briefly, human fetal bone is isolated and a longitudinally sliced portion of this bone is transferred into the mammary fat pad of a SCID/SCID animal. The bone cavity is diminished in endogenous cells by whole body irradiation of the mouse host prior to injection of the test  
25 donor hematopoietic cell population. The HLA of the population which is injected is mismatched with the HLA of the recipient bone cells. Stem cells from human hematopoietic sources will sustain B lymphopoiesis and myelopoiesis in such a SCID-hu bone model.

To demonstrate the ability of the hematopoietic stem cell population to give rise to red blood cells, one may use conventional techniques to identify BFU-E units, for example,  
30 methylcellulose culture to show that the cells are capable of developing the erythroid lineage. See Metcalf (1977) In: Recent Results in Cancer Research 61. Springer-Verlag, Berlin, pp. 1-227.

## Examples

The following examples are included to more fully illustrate the present invention. Additionally, these examples provide preferred embodiments of the invention and are not meant to limit the scope thereof. Standard methods for many of the procedures described in the following examples, or suitable alternative procedures, are provided in widely reorganized manuals of molecular biology, such as, for example "Molecular Cloning," Second Edition (Sambrook, *et al.*, Cold Spring Harbor Laboratory Press, 1987) and "Current Protocols in Molecular Biology" (Ausubel, *et al.*, eds. Greene Associates/Wiley Interscience, NY, 1990).

### EXAMPLE 1

#### PREPARATION OF RETROVIRAL VECTOR BACKBONES

The following example describes the production of three retroviral vector backbones, designated KT-1, KT-3B, KT-3C. Vector KT-1 differs from KT-3B and KT-3C in that the former lacks a selectable marker which in KT-3B is neomycin resistance, whereas KT-3C confers phleomycin resistance.

The Moloney murine leukemia virus (MoMLV) 5' long terminal repeat (LTR) EcoR I-EcoR I fragment, including *gag* sequences, from the N2 vector (Armentano *et al.*, *J. Vir.* 61:1647, 1987; Eglitis *et al.*, *Science* 230:1395, 1985) is ligated into the plasmid SK<sup>+</sup> (Stratagene, La Jolla, CA). The resulting construct is designated N2R5. The N2R5 construct is mutated by site-directed *in vitro* mutagenesis to change the ATG start codon to ATT preventing *gag* expression. This mutagenized fragment is 200 base pairs (bp) in length and flanked by Pst I restriction sites. The Pst I-Pst I mutated fragment is purified from the SK<sup>+</sup> plasmid and inserted into the Pst I site of N2 MoMLV 5' LTR in plasmid pUC31 to replace the non-mutated 200 bp fragment. The plasmid pUC31 is derived from pUC19 (Stratagene, La Jolla, CA) in which additional restriction sites Xho I, Bgl II, BssH II and Nco I are inserted between the EcoR I and Sac I sites of the polylinker. This construct is designated pUC31/N2R5gM.

A 1.0 kilobase (Kb) MoMLV 3' LTR EcoR I-EcoR I fragment from N2 is cloned into plasmid SK<sup>+</sup> resulting in a construct designated N2R3<sup>+</sup>. A 1.0 Kb Cla I-Hind III fragment is purified from this construct.

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The Cla I-Cla I dominant selectable marker gene fragment from pAFVXM retroviral vector (Kriegler *et al.*, *Cell* 38:483, 1984; St. Louis *et al.*, *PNAS* 85:3150, 1988), comprising a SV40 early promoter driving expression of the neomycin (neo) phosphotransferase gene, is cloned into the SK<sup>+</sup> plasmid. This construct is designated SK<sup>+</sup> SV<sub>2</sub>-neo. A 1.3 Kb Cla I-BstB I gene fragment is purified from the SK<sup>+</sup> SV<sub>2</sub>-neo plasmid.

KT-3B or KT-1 vectors are constructed by a three part ligation in which the Xho I-Cla I fragment containing the gene of interest and the 1.0 Kb MoMLV 3' LTR Cla I-Hind III fragment are inserted into the Xho I-Hind III site of pUC31/N2R5gM plasmid. This gives a vector designated as having the KT-1 backbone. The 1.3 Kb Cla I-BstB I neo gene fragment from the pAFVXM retroviral vector is then inserted into the Cla I site of this plasmid in the sense orientation to yield a vector designated as having the KT-3B backbone.

An alternative selectable marker, phleomycin resistance (Mulsant, *et al.*, *Som. Cell and Mol. Gen.*, 14:243, 1988, available from Cayla, Cedex, FR) is used to make the retroviral backbone KT-3C as follows. The plasmid pUT507 (Mulsant, *et al.*, *supra*) is digested with Nde I and the ends blunted with Klenow polymerase I. The sample is then further digested with Hpa I, Cla I linkers ligated to the mix of fragments, followed by digestion with Cla I to remove excess Cla I linkers. The 1.2 Kb Cla I fragment carrying the RSV LTR and the phleomycin resistance gene is isolated by agarose gel electrophoresis followed by purification using Gene Clean (Bio101, San Diego, CA). This fragment is used in place of the 1.3 Kb Cla I-BstB I neomycin resistance fragment to give the backbone KT-3C.

## EXAMPLE 2

### PREPARATION OF RETROVIRAL VECTOR CONSTRUCTS ENCODING PROTEINS

The following example describes the preparation of various retroviral vector constructs encoding different human genes of interest. More specifically, part (A) describes the production of a vector construct encoding the marker gene  $\beta$  galactosidase from *E. coli*, part (B) human interferon (hIFN), part (C) a retroviral vector construct encoding human interleukin-2 (hIL-2), and part (D) the production of two retroviral vector constructs coding for human factor VIII. The first factor VIII construct, codes for the B domain deleted form of the protein, while the second construct codes for full length factor VIII.

A. Preparation of C $\beta$ -gal

$\beta$ -gal is obtained from the plasmid pSP65 as a HindIII-SmaI fragment.

5 B. Preparation of KT-rhy-IFN

To obtain the human  $\gamma$ -IFN gene, the murine homologue is first cloned as follows: A my-IFN cDNA is cloned into the EcoR I site of pUC1813 essentially as set forth below. Briefly, pUC1813 (containing a sequence encoding  $\gamma$ -IFN) is prepared as essentially  
10 described by Kay *et al.*, *Nucleic Acids Research* 15:2778, 1987; and Gray *et al.*, *PNAS* 80:5842, 1983) (Figure 1A). The my-IFN cDNA is retrieved by EcoR I digestion of pUC1813, and the isolated fragment is cloned into the EcoR I site of phosphatase-treated pSP73 (Promega; Madison, WI). This construct is designated SP my-IFN. The orientation of the cDNA is verified by appropriate restriction enzyme digestion and DNA sequencing. In  
15 the sense orientation, the 5' end of the cDNA is adjacent to the Xho I site of the pSP73 polylinker and the 3' end adjacent to the Cla I site. The Xho I-Cla I fragment containing the my-IFN cDNA in either sense or antisense orientation is retrieved from SP my-IFN construct and cloned into the Xho I-Cla I site of the KT-3 retroviral backbone. This construct is designated KT my-IFN.

20

1. Preparation Of Sequences Encoding hy-IFN Utilizing PCR

(a) *PHA Stimulation Of Jurkat Cells*

25 Jurkat cells (ATCC No. CRL 8163) are resuspended at a concentration of  $1 \times 10^6$  cells/ml in RPMI growth media (Irvine Scientific; Santa Ana, CA) with 5% fetal bovine serum (FBS) to a final volume of 158.0 ml. Phytohemagglutinin ("PHA") (Curtis Mathes Scientific, Houston, TX) is added to the suspension to a final concentration of 1%. The suspension is incubated at 37°C in 5% CO<sub>2</sub> overnight. The cells are harvested on the  
30 following day and aliquoted into three 50.0 ml centrifuge tubes. The three pellets are combined in 50 ml 1x phosphate buffered saline (PBS, 145 mM, pH 7.0) and centrifuged at 1000 rpm for 5 minutes. The supernatant is decanted and the cells are washed with 50.0 ml PBS. The cells are collected for RNA isolation.

(b) *RNA Isolation*

The PHA stimulated Jurkat cells are resuspended in 22.0 ml guanidinium solution (4 M guanidinium thiocyanate; 20 mM sodium acetate, pH 5.2; 0.1 M dithiothreitol, 0.5% sarcosyl). This cell-guanidinium suspension is then passed through a 20 gauge needle six times in order to disrupt cell membranes. A CsCl solution (5.7 M CsCl, 0.1 M EDTA) is then overlaid with 11.0 mL of the disrupted cell-guanidinium solution. The solution is centrifuged for 24 hours at 28,000 rpm in a SW28.1 rotor (Beckman, Fullerton, CA) at 20°C. After centrifugation the supernatant is carefully aspirated and the tubes blotted dry. The pellet is resuspended in a guanidinium-HCl solution (7.4 M guanidinium-HCl; 25 mM Tris-HCl, pH 7.5; 5 mM dithiothreitol) to a final volume of 500.0 µl. This solution is transferred to a microcentrifuge tube. Twelve and one-half microliters of concentrated Glacial acetic acid (HAc) and 250 µl of 100% EtOH are added to the microfuge tube. The solution is mixed and stored for several days at -20°C to precipitate RNA.

After storage, the solution is centrifuged for 20 minutes at 14,000 rpm, 4°C. The pellet is then resuspended in 75% EtOH and centrifuged for 10 minutes in a microfuge at 14,000 rpm, 4°C. The pellet is dried by centrifugation under vacuum, and resuspended in 300 L deionized (DI) H<sub>2</sub>O. The concentration and purity of the RNA is determined by measuring optical densities at 260 and 280 nm.

(c) *Reverse Transcription Reaction*

Immediately before use, 5.0 l (3.4 mg/mL) of purified Jurkat RNA is heat treated for 5 minutes at 90°C, and then placed on ice. A solution of 10.0 µl of 10x PCR buffer (500 mM KCl; 200 mM Tris-HCl, pH 8.4; 25 mM MgCl<sub>2</sub>; 1 mg/ml bovine serum albumin (BSA)); 10.0 µl of 10 mM dATP, 10.0 µl of 10 mM dGTP, 10.0 µl of 10 mM dCTP, 10.0 µl of 10 mM dTTP, 2.5 µl RNasin (40,000 U/ml, Promega; Madison, WI) and 33.0 µl DI H<sub>2</sub>O, is added to the heat treated Jurkat cell RNA. To this solution 5.0 µl (10<sup>8</sup> nmol/mL) (Sequence ID No. 1), and 5.0 µl (200,000 U/ml) MoMLV reverse transcriptase (Bethesda Research Laboratories, EC 3.1.27.5, MD) is mixed in a microfuge tube and incubated at room temperature for 10 minutes. Following the room temperature incubation, the reaction mixture is incubated for 1 hour at 37°C, and then incubated for 5 minutes at 95°C. The reverse transcription reaction mixture is then placed on ice in preparation for PCR.



(d) *PCR Amplification*

The PCR reaction mixture contains 100.0 µl reverse transcription reaction; 356.0 µl DI H<sub>2</sub>O; 40.0 µl 10x PCR buffer; 1.0 µl (137 nmol/mL) V-OLI #5 (Sequence ID No. 2); 0.5 µl (320 nmol/mL) V-OLI #6 (Sequence ID No. 3), and 2.5 µl, 5,000 U/ml, Taq polymerase (EC 2.7.7.7, Perkin-Elmer Cetus, CA). One hundred microliters of this mixture is aliquoted into each of 5 tubes.

(Sequence ID No. 1)

10 5' - 3': TAA TAA ATA GAT TTA GAT TTA

This primer is complementary to a sequence of the my-IFN cDNA 30 base pairs downstream of the stop codon.

V (Sequence ID No. 2)

15 5' - 3': GC CTC GAG ACG ATG AAA TAT ACA AGT TAT ATC TTG

This primer is complementary to the 5' coding region of the my-IFN gene, beginning at the ATG start codon. The 5' end of the primer contains a Xho I restriction site.

(Sequence ID No. 3)

20 5' - 3': GA ATC GAT CCA TTA CTG GGA TGC TCT TCG ACC TGG

This primer is complementary to the 3' coding region of the my-IFN gene, ending at the TAA stop codon. The 5' end of the primer contains a Cla I restriction site.

Each tube was overlaid with 100.0 µl mineral oil, and placed into a PCR machine (Ericomp Twin Block System, Ericomp, CA). The PCR program regulates the temperature of the reaction vessel first at 95°C for 1 minute, next at 67°C for 2 minutes and finally at 72°C for 2 minutes. This cycle is repeated 40 times. The last cycle regulates the temperature of the reaction vessel first at 95°C for 1 minute, next at 67°C for 2 minutes and finally at 72°C for 7 minutes. The completed PCR amplification reactions are stored at 4°C for 1 month in preparation for PCR DNA isolation.

(e) *Isolation Of PCR DNA*

35 The aqueous phase from the PCR amplification reactions are transferred into a single microfuge tube. Fifty microliters of 3 M sodium acetate and 500.0 µl of chloroform:isoamyl

alcohol (24:1) is added to the solution. The solution is vortexed and then centrifuged at 14,000 rpm at room temperature for 5 minutes. The upper aqueous phase is transferred to a fresh microfuge tube and 1.0 mL of 100% EtOH is added. This solution is incubated for 4.5 hours at -20°C and then centrifuged at 14,000 rpm for 20 minutes. The supernatant is  
5 decanted, and the pellet is rinsed with 500.0 µl of 70% EtOH. The pellet is dried by centrifugation under a vacuum. The isolated hy-IFN PCR DNA is resuspended in 10.0 µl DI H<sub>2</sub>O.

## 10            2.        Construction Of h-IFN Retroviral Vectors

### (a)        *Creation And Isolation Of Blunt-Ended hg-IFN PCR DNA Fragments*

The hy-IFN PCR DNA is blunt ended using T4 DNA polymerase. Specifically, 10.0  
15 µl of PCR amplified DNA; 2.0 µl, 10x, T4 DNA polymerase buffer (0.33 M Tris-acetate, pH 7.9, 0.66 M potassium acetate, 0.10 M magnesium acetate, 5 mM dithiothreitol, 1 mg/mL bovine serum albumin (BSA)); 1.0 µl, 2.5 mM dNTP (a mixture containing equal molar concentrations of dATP, dGTP, dTTP and dCTP); 7.0 µl DI H<sub>2</sub>O; 1.0 µl, 5000 U/mL, Klenow fragment (EC 2.7.7.7, New England Biolabs, MA); and 1.0 µl, 3000 U/mL, T4 DNA  
20 polymerase (EC 2.7.7.7, New England Biolabs, MA) are mixed together and incubated at 37°C for 15 minutes. The reaction mixture is then incubated at room temperature for 40 minutes and followed by an incubation at 68°C for 15 minutes.

The blunt ended hy-IFN is isolated by agarose gel electrophoresis. Specifically, 2.0  
25 µl of loading dye (0.25% bromophenol blue; 0.25% xylene cyanol; and 50% glycerol) is added to reaction mixture and 4.0 µl is loaded into each of 5 lanes of a 1% agarose/Tris-borate-EDTA (TBE) gel containing ethidium bromide. Electrophoresis of the gel is performed for 1 hour at 100 volts. The desired DNA band containing hy-IFN, approximately 500 base pairs in length, is visualized under ultraviolet light.

This band is removed from the gel by electrophoretic transfer onto NA 45 paper  
30 (Schleicher and Schuell, Keene, NH. The paper is incubated at 68°C for 40 minutes in 400.0 µl of high salt NET buffer (1 M NaCl; 0.1 mM EDTA; and 20 mM Tris, pH 8.0) to elute the DNA. The NA 45 paper is removed from solution and 400.0 µl of phenol:chloroform:isoamyl alcohol (25:24:1) is added. The solution is vortexed and centrifuged at 14,000 for 5 minutes. The upper aqueous phase is transferred to a fresh tube  
35 and 400.0 µl of chloroform:isoamyl alcohol (24:1) is added. The mixture is vortexed and

centrifuged for 5 minutes. The upper aqueous phase is transferred, a second time, to a fresh tube and 700.0  $\mu$ l of 100% EtOH is added. The tube is incubated at -20°C for 3 days.

Following incubation, the DNA is precipitated from the tube by centrifugation for 20 minutes at 14,000 rpm. The supernatant is decanted and the pellet is rinsed with 500.0  $\mu$ l of 70% EtOH. The pellet, containing blunt ended *hy*-IFN DNA, is dried by centrifugation under vacuum and resuspended in 50.0  $\mu$ l of DI H<sub>2</sub>O.

The isolated blunt ended *hy*-IFN DNA is phosphorylated using polynucleotide kinase. Specifically, 25.0  $\mu$ l of blunt-ended *hy*-IFN DNA, 3.0  $\mu$ l of 10x kinase buffer (0.5 M Tris-HCl, pH 7.6; 0.1 M MgCl<sub>2</sub>; 50 mM dithiothreitol; 1 mM spermidine; 1 mM EDTA), 3.0  $\mu$ l of 10 mM ATP, and 1.0  $\mu$ l of T4 polynucleotide kinase (10,000 U/ml, EC 2.7.1.78, New England Biolabs, MD) is mixed and incubated at 37°C for 1 hour 45 minutes. The enzyme is then heat inactivated by incubating at 68°C for 30 minutes.

(b) *Ligation Of hy-IFN PCR DNA Into The SK<sup>+</sup> Vector*

An SK<sup>+</sup> plasmid is digested with Hinc II restriction endonuclease and purified by agarose gel electrophoresis as described below. Specifically, 5.9  $\mu$ l (1.7 mg/mL) SK<sup>+</sup> plasmid DNA (Stratagene, San Diego, CA); 4.0  $\mu$ l 10x Universal buffer (Stratagene, San Diego, CA); 30.1  $\mu$ l DI H<sub>2</sub>O, and 4.0  $\mu$ l Hinc II, 10,000 U/mL, are mixed in a tube and incubated for 7 hours at 37°C. Following incubation, 4.0  $\mu$ l of loading dye is added to the reaction mixture and 4.0  $\mu$ l of this solution is added to each of 5 lanes of a 1% agarose/TBE gel containing ethidium bromide. Electrophoresis of the gel is performed for 2 hours at 105 volts. The Hinc II cut SK<sup>+</sup> plasmid, 2958 base pairs in length, is visualized with ultraviolet light. The digested SK<sup>+</sup> plasmid is isolated by gel electrophoresis.

Dephosphorylation of the Hinc II cleavage site of the plasmid is performed using calf intestine alkaline phosphatase. Specifically, 50.0  $\mu$ l digested SK<sup>+</sup> plasmid; 5.0  $\mu$ l 1 M Tris, pH 8.0; 2.0  $\mu$ l 5 mM EDTA, pH 8.0; 43.0  $\mu$ l H<sub>2</sub>O and 2.0  $\mu$ l, 1,000 U/mL, calf intestinal phosphatase ("CIP") (Boehringer Mannheim, Indianapolis, IN) are mixed in a tube and incubated at 37°C for 15 minutes. Following incubation, 2.0  $\mu$ l CIP is added. and the solution is incubated at 55°C for 90 minutes. Following this incubation, 2.5  $\mu$ l 20% sodium dodecyl sulfate ("SDS"), 1.0  $\mu$ l 0.5 M EDTA, pH 8.0, and 0.5  $\mu$ l, 20 mg/mL, proteinase K (EC 3.4.21.14, Boehringer Mannheim, Indianapolis, IN) are added, and the solution is incubated at 55°C for 2 hours. This solution is cooled to room temperature, and 110.0  $\mu$ l phenol:chloroform:isoamyl alcohol (25:24:1) is added. The mixture is vortexed and centrifuged at 14,000 rpm for 5 minutes. The upper aqueous phase is transferred to a fresh

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tube and 200.0  $\mu$ l of 100% EtOH is added. This mixture is incubated at 70°C for 15 minutes. The tube is centrifuged and the pellet is rinsed with 500.0  $\mu$ l of 70% EtOH. The pellet was then dried by centrifugation under a vacuum. The dephosphorylated SK<sup>+</sup> plasmid is resuspended in 40  $\mu$ l DI H<sub>2</sub>O.

- 5           The hy-IFN PCR DNA is ligated into the SK<sup>+</sup> plasmid using T4 DNA ligase. Specifically, 30.0  $\mu$ l blunt ended, phosphorylated, hy-IFN PCR DNA reaction mixture, 2.0  $\mu$ l dephosphorylated SK<sup>+</sup> plasmid and 1.0  $\mu$ l T4 DNA ligase are combined in a tube and incubated overnight at 16°C. DNA was isolated using a minprep procedure. More specifically, the bacterial strain DH5a (Gibco BRL, Gaithersburg, MD) is transformed with
- 10   15.0  $\mu$ l of ligation reaction mixture, plated on Luria-Bertani agar plates (LB plates) containing ampicillin and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal, Gold Biotechnology, St. Louis, MO), and incubated overnight at 37°C. DNA is isolated from white bacterial colonies using the procedure described by Sambrook *et al.* (*Molecular Cloning*, Cold Springs Harbor Press, 1989). The presence of the hy-IFN gene is determined
- 15   by restriction endonuclease cleavage with Xho I, Cla I, Ava II, Dra I, and Ssp I. The expected endonuclease restriction cleavage fragment sizes for plasmids containing the hy-IFN gene are presented in Table 2. The isolated DNA plasmid is designated SK hy-IFN and used in constructing the retroviral vectors.

20

Table 2

Enzyme	Fragment Size (bp)
Xho I and Cla I	500, 2958
Ava II	222, 1307, 1937
Dra I	700, 1149, 1500
Ssp I	750, 1296, 2600

25

30

(c) *Ligation Of hy-IFN Gene Into Retroviral Vector*

The interferon gene is removed from SK hy-IFN vector by digestion with Xho I and Cla I restriction endonucleases. The resulting fragment containing the hy-IFN gene is approximately 500 bp in length, and is isolated in a 1% agarose/TBE gel electrophoresis. The Xho I-Cla I hy-IFN fragment is then ligated into the KT-3 retroviral backbone. This construct is designated KT hy-IFN. The structure and presence expression of hy-IFN is determined by transforming DH5a bacterial strain with the KT hy-IFN construct. Specifically, the bacteria is transformed with 15.0 µl of ligation reaction mixture. The transformed bacterial cells are plated on LB plates containing ampicillin. The plates are incubated overnight at 37°C and bacterial colonies are selected. The DNA is isolated as described in (b) above, and digested with Xho I, Cla I, Dra I, Nde I, and Ssp I. The expected endonuclease restriction cleavage fragment sizes for plasmids containing the hy-IFN gene are presented in Table 3.

Table 3

Enzyme	Fragment Size (bp)
Xho I and Cla I	500, 6500
Nde I	1900, 5100
Dra I	692, 2700, 3600
Ssp I	541, 1700, 4700

Subsequent sequencing of KT hy-IFN, the retroviral vector, revealed the presence of a one base pair deletion within the hy-IFN gene. This deletion is reversed using multi-step PCR procedure.

i. Sequence Selection

Sequences are obtained from IBI Pustell sequence analysis program (Int Biotech, Inc., New Haven, CT).

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The following hy-IFN primer sequences are used:

(Sequence ID No. 4)

5'-3': G CCT CGA GCT CGA GCG ATG AAA TAT ACA AGT TAT ATC TTG

- 5 This primer is the sense sequence complimentary to the start codon ATG region extending seven codons upstream of hy-IFN gene, and is designated hy-IFN 1b.

(Sequence ID No. 5)

5'-3': GTC ATC TCG TTT CTT TTT GTT GCT ATT

- 10 This primer is the anti-sense sequence complimentary to codons 106 to 120 of the hy-IFN gene, and is designated hy-IFN Rep B.

(Sequence ID No. 6)

5'-3': AAT AGC AAC AAA AAG AAA CGA GAT GAC

- 15 This primer is the sense sequence complimentary to codons 106 to 120 of the hy-IFN gene, and is designated hy-IFN Rep A.

(Sequence ID No. 7)

5'-3': G CAT CGA TAT CGA TCA TTA CTG GGA TGC TCT TCG ACC TCG

- 20 This primer is the anti-sense sequence complimentary to the stop codon ATT region and extending seven codons upstream of the hy-IFN gene, and is designated hy-IFN 3b.

## ii. Initial PCR

25

- A solution of  $1 \times 10^6$  KT hy-IFN plasmid molecules in 398.0  $\mu$ l, DI H<sub>2</sub>O; 50  $\mu$ l, 10x PCR buffer (500 mM KCl and 200 mM Tris-HCl, pH 8.4; 25 mM MgCl<sub>2</sub>; 1.0 mg/ml BSA); 5.0  $\mu$ l, 2.5 mM dATP; 5.0  $\mu$ l, 2.5 mM dGTP; 5.0  $\mu$ l, 2.5 mM dCTP; 5.0  $\mu$ l, 2.5 mM dTTP; 12.0  $\mu$ l, 18.6 nmol/ml, oligonucleotide hy-IFN 1b; 15.0  $\mu$ l, 24.6 nmol/ml, oligonucleotide hy-IFN RepB; and 2.5  $\mu$ l, Taq polymerase is mixed in a microfuge tube and 50  $\mu$ l is aliquoted into 10 tubes. Similarly, a solution of  $1 \times 10^6$  KT hy-IFN plasmid molecules in 395.0  $\mu$ l, DI H<sub>2</sub>O; 50.0  $\mu$ l, 10x PCR buffer (500 mM KCl; 200 mM Tris-HCl, pH 8.4; 25 mM MgCl<sub>2</sub>; 1 mg/ml BSA); 5.0  $\mu$ l, 2.5 mM dATP; 5.0  $\mu$ l, 2.5 mM dGTP; 5.0  $\mu$ l, 2.5 mM dCTP; 5.0  $\mu$ l, 2.5 mM dTTP; 13  $\mu$ l, 23.4 nmol/ml, oligonucleotide hy-IFN RepA; 17.0  $\mu$ l, 18.0 nmol/ml, oligonucleotide hy-IFN 3b; and 2.5  $\mu$ l Taq polymerase is mixed in a microfuge tube and 50.0
- 30
- 35

5  $\mu$ l is aliquoted into 10 tubes. The 20 tubes are placed in a PCR machine (Model 9600, Perkin Elmer Cetus; Los Angeles, CA). The PCR program regulates the temperature of the reaction vessel in the first cycle at 94°C for 2 minutes. The next 35 cycles are regulated at 94°C for 0.5 minutes, then at 55°C for 0.5 minutes and finally at 72°C for 1 minute. The final cycle is regulated at 72°C for 10 minutes. This cycling program is designated Program 10.

10 Following PCR amplification, 225.0  $\mu$ l of each reaction tube is mixed with 25.0  $\mu$ l loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 50% glycerol, agarose gel loading dye) and loaded into the wells of a 2% agarose gel containing ethidium bromide. The gel is electrophoresed at approximately 90 volts for 1 hour. Ultraviolet light is used to visualize the DNA band separation. Two bands are isolated, one fragment of 250 bp in size and the other of 150 bp in size by electrophoretic transfer onto NA 45 paper. Following precipitation, each of the two DNA pellets is resuspended in 20.0 l DI H<sub>2</sub>O and prepared for further PCR amplification.

15

iii. Annealing and Second Round PCR

20 A solution of 20.0  $\mu$ l of the 150 bp PCR DNA; 20.0  $\mu$ l of the 350 bp PCR DNA; 161.5  $\mu$ l, DI H<sub>2</sub>O; 25.0  $\mu$ l, 10x PCR buffer (500 mM KCl; 200 mM Tris-HCl, pH 8.4; 25 mM MgCl<sub>2</sub>; and 1 mg/ml BSA); 2.5  $\mu$ l, 2.5 mM dATP; 2.5  $\mu$ l, 2.5 mM dGTP; 2.5  $\mu$ l, 2.5 mM dCTP; 2.5  $\mu$ l, 2.5 mM dTTP; and 1.25  $\mu$ l Taq polymerase is mixed in a microfuge tube and 47.3  $\mu$ l aliquoted into each of 5 tubes. Each tube is placed in a PCR machine (Model 9600, Perkin-Elmer-Cetus, CA). The PCR program regulates the temperature of the reaction vessel for 5 cycles at 94°C for 0.5 minutes. The next cycle is regulated at 55°C for 1 minute. Following this cycle, 1.2  $\mu$ l hy-IFN 1b and 1.5  $\mu$ l hy-IFN 3b are added to the reaction mixture. The tubes are then PCR amplified using program 10. The product is designated rhy-IFN.

30

iv. Creation and Isolation of Blunt-Ended rhg-IFN PCR DNA Fragment

35 The PCR amplified hy-IFN DNA is blunt ended using T4 polymerase. Specifically, 120.0  $\mu$ l rhy-IFN PCR solution is mixed with 1.25  $\mu$ l, 2.5 mM dATP; 1.25  $\mu$ l, 2.5 mM dGTP; 1.25  $\mu$ l, 2.5 mM dCTP; 1.25 l, 2.5 mM dTTP; 1 l, T4 DNA polymerase; and 1.0  $\mu$ l

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Klenow fragment. This mixture is incubated at room temperature for 10 minutes. Following incubation, 13.0  $\mu$ l of agarose gel loading dye is added to the mixture and this solution is loaded into a 1% agarose gel. The gel is electrophoresed at approximately 90 volts for 1 hour. Ultraviolet light is used to visualize the DNA banding. A 500 bp band is isolated by electrophoretic transfer onto NA 45 paper as described above. Following precipitation, the DNA pellet is resuspended in 12.0 l DI H<sub>2</sub>O.

The isolated 500 bp fragment is blunt ended using T4 polynucleotide kinase. Specifically, 1.0 mg of this fragment is mixed with 1.5  $\mu$ l 10x kinase buffer (0.5 mM Tris-HCl, pH 7.6; 0.1 mM MgCl<sub>2</sub>; 50 mM dithiothreitol; 1 mM spermidine; 1 mM EDTA); 1.5  $\mu$ l, 10 mM ATP; and 1.0  $\mu$ l, T4 polynucleotide kinase, and incubated at 37°C for 30 minutes.

v. Ligation of rhy-IFN PCR DNA Into the SK<sup>+</sup> Vector

The rhy-IFN PCR DNA is ligated into the SK<sup>+</sup> vector. A solution of 2.0  $\mu$ l hy-IFN PCR DNA-kinase reaction mixture; 2.0  $\mu$ l CIP treated SK<sup>+</sup> vector; and 1.0  $\mu$ l, T4 DNA ligase is incubated at 16°C for 4 hours. DH5a bacteria is transformed as described above.

vi. Ligation of hy-IFN Gene Into Retroviral Vector

20

Ligation of hy-IFN gene into retroviral vector is performed as described above. The new vector is designated KT hy-IFN.

C. Preparation of KT-hIL-2.

25

The method for cloning hIL-2 into KT-3 retroviral vector is essentially identical to the procedure for cloning hg-IFN into KT-3, with the exception that different primers are required for amplification of the hIL-2 DNA sequence. The following hIL-2 PCR primer sequences are used:

30

V-OLI #55 (Sequence ID No. 8)

5'-3': ATA AAT AGA AGG CCT GAT ATG

This primer is complimentary to a sequence of the hIL-2 cDNA downstream of the stop codon.

35



## V-OLI #1 (Sequence ID No. 9)

5'-3': GC CTC GAG ACA ATG TAC AGG ATG CAA CTC CTG TCT

This primer is the sense sequence of the hIL-2 gene complimentary to the 5' coding region beginning at the ATG start codon. The 5' end of the primer contains a Xho I  
5 restriction site.

## V-OLI #2 (Sequence ID No. 10)

5'-3': GA ATC GAT TTA TCA AGT CAG TGT TGA GAT GAT GCT

The primer is the anti-sense sequence of the hIL-2 gene complimentary to the 3' coding region ending at the TAA stop codon. The 5' end of the primer contains the Cla I  
10 restriction site.

D. Preparation of Factor VIII Vectors.

15 The following is a description of the construction of several retroviral vectors encoding factor VIII. Due to the size of the full length factor VIII gene (7,056 bp), packaging constraints of retroviral vectors and because selection for transduced cells is not a requirement for ex vivo hematopoietic stem cell therapy, a retroviral backbone, *e.g.*, KT-1,  
20 lacking a selectable marker gene is employed.

A gene encoding full length factor VIII can be obtained from a variety of sources. One such source is the plasmid pCIS-F8 (EP 0 260 148 A2, published March 3, 1993), which contains a full length factor VIII cDNA whose expression is under the control of a CMV major immediate-early (CMV MIE) promoter and enhancer. The factor VIII cDNA  
25 contains approximately 80 bp of 5' untranslated sequence from the factor VIII gene and a 3' untranslated region of about 500 bp. In addition, between the CMV promoter and the factor VIII sequence lies a CMV intron sequence, or "cis" element. The cis element, spanning about 280 bp, comprises a splice donor site from the CMV major immediate-early promoter about 140 bp upstream of a splice acceptor from an immunoglobulin gene, with the  
30 intervening region being supplied by an Ig variable region intron.

i. Construction of a Plasmid Encoding Retroviral Vector JW-2.

A plasmid, pJW-2, encoding a retroviral vector for expressing full length factor VIII  
35 is constructed using the KT-1 backbone from pKT-1. To facilitate directional cloning of the

factor VIII cDNA insert into pKT-1, the unique Xho I site is converted to a Not I site by site directed mutagenesis. The resultant plasmid vector is then opened with Not I and Cla I. pCIS-F8 is digested to completion with Cla I and Eag I, for which there are two sites, to release the fragment encoding full length factor VIII. This fragment is then ligated into the  
5 Not I/Cla I restricted vector to generate a plasmid designated pJW-2.

ii. Construction of a Plasmid Encoding Retroviral Vector ND-5

10 A plasmid vector encoding a truncation of about 80% (approximately 370 bp) of the 3' untranslated region of the factor VIII cDNA, designated pND-5, is constructed in a pKT-1 vector as follows: As described for pJW-2, the pKT-1 vector employed has its Xho I restriction site replaced by that for Not I. The factor VIII insert is generated by digesting pCIS-F8 with Cla I and Xba I, the latter enzyme cutting 5' of the factor VIII stop codon.  
15 The approximately 7 kb fragment containing all but the 3' coding region of the factor VIII gene is then purified. pCIS-F8 is also digested with Xba I and Pst I to release a 121 bp fragment containing the gene's termination codon. This fragment is also purified and then ligated in a three way ligation with the larger fragment encoding the rest of the factor VIII gene and Cla I/Pst I restricted BLUESCRIPT® KS<sup>+</sup> plasmid (Stratagene, San Diego, CA) to  
20 produce a plasmid designated pND-2.

The unique Sma I site in pND-2 is then changed to a Cla I site by ligating Cla I linkers (New England Biolabs, Beverly, MA) under dilute conditions to the blunt ends created by a Sma I digest. After recircularization and ligation, plasmids containing two Cla I sites are identified and designated pND-3.

25 The factor VIII sequence in pND-3, bounded by Cla I sites and containing the full length gene with a truncation of much of the 3' untranslated region, is cloned as follows into a plasmid backbone derived from a Not I/Cla I digest of pJW-1 [a pKT-1 derivative by cutting at the Xho I site, blunting with Klenow, and inserting a Not I linker (New England Biolabs)], which yields a 5.2 kb Not I/Cla I fragment. pCIS-F8 is cleaved with Eag I and  
30 Eco RV and the resulting fragment of about 4.2 kb, encoding the 5' portion of the full length factor VIII gene, is isolated. pND-3 is digested with Eco RV and Cla I and a 3.1 kb fragment is isolated. The two fragments containing portions of the factor VIII gene are then ligated into the Not I/Cla I digested vector backbone to produce a plasmid designated pND-  
5.

35

iii. Construction of a B Domain-deleted Factor VIII Vector

The precursor DNA for the B-deleted FVIII is obtained from Miles Laboratory. This expression vector is designated p25D and has the exact backbone as pCISF8 above. The Hpa I site at the 3' of the FVIII8 cDNA in p25D is modified to Cla-I by oligolinkers. An Acc I to Cla I fragment is clipped out from the modified p25D plasmid. This fragment spans the B-domain deletion and includes the entire 3' two-thirds of the cDNA. An Acc I to Cla I fragment is removed from the pJW-2 above, and replaced with the modified B-domain deleted fragment just described. This construct is designated B-del-1.

As those in the art will appreciate, after construction of plasmids encoding retroviral vectors such as those described above, such plasmids can then be used in the production of various cell lines from which infectious recombinant retroviruses can be produced.

E. Preparation of MDR-1

Plasmid clones containing the the multi-drug resistance-1 (MDR-1) gene were obtained from ATCC (ATCC No. 61360 and 65704). The gene is isolated and purified using methods provided in Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2d., 1989). Appropriate endonuclease restriction sites, Xho I and Cla I, (*Molecular Cloning: A Laboratory Manual*, 2d., 1989) are provided for insertion of the MDR-1 gene into KT-1 or KT-3B retroviral vector backbones.

EXAMPLE 3

Isolation and Transduction of Bone Marrow Cells

Pluripotent hematopoietic stem cells, CD34<sup>+</sup> are collected from the bone marrow of a patient by a syringe evacuation performed by known techniques. Alternatively, CD34<sup>+</sup> cells may also be obtained from the cord blood of an infant if the patient is diagnosed before birth. Generally, 20 bone-marrow aspirations are obtained by puncturing femoral shafts or from the posterior iliac crest under local or general anesthesia. Bone marrow aspirations are then pooled and suspended in Hepes-buffered Hanks' balanced salt solution containing heparin sulfate at 100 Units/ml and deoxyribonuclease I at 100 µg/ml and then subjected to a Ficoll gradient separation. The buffy coated marrow cells are then collected and washed

according to CEPRATE™ LC (CD34) Separation system (Cellpro, Bothell, WA). The washed buffy coated cells are then stained sequentially with anti-CD34 monoclonal antibody, washed, then stained with biotinylated secondary antibody supplied with the CEPRATE™ system. The cell mixture is then loaded onto the CEPRATE™ avidin column. The biotin-labeled cells are adsorbed onto the column while unlabeled cells pass through. The column is then rinsed according to the CEPRATE™ system directions and CD34<sup>+</sup> cells eluted by agitation of the column by manually squeezing the gel bed. Once the CD34<sup>+</sup> cells are purified, the purified stem cells are counted and plated at a concentration of  $1 \times 10^5$  cells/ml in Iscove's modified Dulbecco's medium, IMDM (Irvine Scientific, Santa Ana, CA), containing 20% pooled non-heat inactivated human AB serum (hAB serum).

After purification of CD34<sup>+</sup> cells, several methods of transducing purified stem cells may be performed. One approach involves transduction of the purified stem cell population with vector containing supernatant cultures derived from vector producing cells. A second approach involves co-cultivation of an irradiated monolayer of vector producing cells with the purified population of non-adherent CD34<sup>+</sup> cells. A third approach involves a similar co-cultivation approach, however the purified CD34<sup>+</sup> cells are pre-stimulated with various cytokines and cultured 48 hours prior to the co-cultivation with the irradiated vector producing cells. Pre-stimulation prior to transduction increases effective gene transfer (Nolta *et al.*, *Exp. Hematol.* 20:1065; 1992). The increased level of transduction is attributed to increased proliferation of the stem cells necessary for efficient retroviral transduction. Stimulation of these cultures to proliferate also provides increased cell populations for re-infusion into the patient.

25

#### EXAMPLE 4

##### Packaging Cell Production

###### A. MLV structural gene expression vectors

To decrease the possibility of replication-competent virus being generated by genetic interactions between the MLV proviral vector DNA and the structural genes of the packaging cell line ("PCL"), separate expression vectors, each lacking the viral LTR, were generated to express the *gag/pol* and *env* genes independently. To further decrease the possibility of homologous recombination with MLV vectors and the resultant generation of replication-competent virus, minimal sequences other than the protein coding sequences were used. In

35

order to express high levels of the MLV structural proteins in the host cells, strong transcriptional promoters (CMV early and Ad5 major late promoters) were utilized. An example of the construction of a MoMLV *gag/pol* expression vector pSCV10 follows:

1. The 0.7 Kb *HinCII/XmaIII* fragment encompassing the human cytomegalovirus (CMV) early transcriptional promoter (Boshart, *et al.*, *Cell* 41:521, 1985) was isolated.
2. A 5.3 Kb *PstI*(partial)/*ScaI* fragment from the MoMLV proviral plasmid, MLV-K (Miller, *et al.*, *Mol. Cell Biol.* 5:531, 1985) encompassing the entire *gag/pol* coding region was isolated.
3. A 0.35 Kb *DraI* fragment from SV40 DNA (residues 2717-2363) encompassing the SV40 late transcriptional termination signal was isolated.
4. Using linkers and other standard recombinant DNA techniques, the CMV promoter-MoMLV *gag/pol*-SV40 termination signal was ligated into the bluescript vector SK<sup>+</sup> (Stratagene, San Diego, CA).

An example of the construction of an MLV xenotropic envelope expression vector follows.

1. A 2.2 Kb *NaeI/NheI* fragment containing the coding region of the xenotropic envelope obtained from clone NZB9-1 (O'Neill, *et al.*, *J. Virol.* 53:100, 1985) was isolated.
2. Using linkers and other standard recombinant DNA techniques, the CMV early promoter and SV40 late termination signal described for the *gag/pol* expression above (pSCV10) were ligated in the order CMV promoter-xeno *env*-termination signal (pCMV<sub>xeno</sub>).

#### B. Host Cell Selection

Host cell lines were screened for their ability to efficiently (high titer) rescue a drug resistance retroviral vector A alpha N2 (Armentano, *et al.*, *J. Vir.* 61:1647, 1987; and Eglitis, *et al.*, *Science* 230:1395, 1985) using replication competent retrovirus to produce the *gag/pol* and *env* structural genes ("MA" virus). Titer was measured from confluent monolayers 16 h after a medium change by adding filtered supernatants (0.45 um filters) to 5x10<sup>4</sup> NIH 3T3 TK<sup>-</sup> cells on a 6 cm tissue culture plate in the presence of 4 ug/ml polybrene followed by selection in G418. Among the non-murine cell lines which demonstrated the ability to package MoMLV-based vector with high titre were the cell lines CF2 (canine), D17 (canine), 293 (human), and HT1080 (human). These cell lines are preferred for production of packaging and producer cell lines, although many other cells may be tested and selected by such means.

C. Generation of Packaging Cell Lines

(i) Preparation of *gag/pol* intermediates

- 5 As examples of the generation of *gag/pol* intermediates for PCL production, D17 (ATCC No. CCL-183), 293 (ATCC No. 1573), and HT1080 (ATCC No. CCL 121) cells were co-transfected with 1 ug of the methotrexate resistance vector, pFR400 (Graham and van der Eb, *Virology* 52:456, 1973), and 10 ug of the MoMLV *gag/pol* expression vector, pSCV10 (above) by calcium phosphate co-precipitation (D17 and HT1080, *see* Graham and van der Eb, 10 *supra*), or lipofection (293, *see* Felgner, *et al.*, *Proc. Natl. Acad. Sci., USA* 84:7413, 1987). After selection for transfected cells in the presence of the drugs dipyrimidol and methotrexate, individual drug resistant cell colonies were expanded and analyzed for MoMLV *gag/pol* expression by extracellular reverse transcriptase (RT) activity (modified from Goff, *et al.*, *J. Virol.* 38:239, 1981) and intracellular p30<sup>gag</sup> by Western blot using anti-p30 antibodies (goat 15 antiserum #77S000087 from the National Cancer Institute). This method identified individual cell clones of each cell type which expressed 10-50x higher levels of both proteins compared with that of the packaging cell line PA317, as shown in Table 4.

TABLE 4  
PROPERTIES OF MoMLV GAG/POL-EXPRESSING CELLS

5	CELL NAME	RT	p30 <sup>gag</sup>	LARNL
		ACTIVITY (CPM)	EXPRESSION	TITRE (CFU/ML)
10	3T3	800	-	N.D.
	PA317	1350	+/-	1.2 x 10 <sup>3</sup>
	D17	800	-	N.D.
	D17 4-15	5000	+++++	1.2 X 10 <sup>4</sup>
	D17 9020	2000	+++	6.0 X 10 <sup>3</sup>
	D17 9-9	2200	++	1.0 X 10 <sup>3</sup>
15	D17 9-16	6100	+++++	1.5 X 10 <sup>4</sup>
	D17 8-7	4000	-	N.D.
	HT1080	900	-	N.D.
	HTSCV21	16400	+++++	8.2 X 10 <sup>3</sup>
	HTSCV25	7900	+++	2.8 X 10 <sup>3</sup>
	HTSCV42	11600	++	8.0 X 10 <sup>2</sup>
20	HTSCV26	4000	-	< 10
	293	600	-	N.D.
	293 2-3	6500	+++++	7 x 10 <sup>4</sup>
	293 5-2	7600	+++++	N.D.

25 The biological activity of these proteins was tested by introducing a retroviral vector, LARNL which expresses both the amphotropic envelope and a Neo<sup>+</sup> marker which confers resistance to the drug G418. In every case, co-expression of *gag/pol* in the cell line and *env* from the vector allowed efficient packaging of the vector as determined by cell-free transfer of G418 resistance to 3T3 cells (titer). Titer was measured from confluent monolayers 16 h after a medium change by adding filtered supernatants (0.45 µm filters) to 5x10<sup>4</sup> NIH353 TK<sup>+</sup> cells on a 6 cm tissue culture plate in the presence of 4 ug/ml polybrene followed by selection in G418.

30 Significantly, the vector titers from the cell lines correlated with the levels of p30<sup>gag</sup> (Table 4). Since the level of *env* should be the same in each clone and is comparable to the level found in PA317 (data not shown), this indicates that titre was limited by the lower levels of *gag/pol* in

these cells (including PA317). The titre correlated more closely with the levels of p30<sup>gag</sup> than with the levels of RT.

(ii) Conversion of gag/pol lines into xenotropic packaging cell lines.

5

As examples of the generation of xenotropic PCLs, the *gag/pol* over-expressors for D17 (4-15) and HT1080 (SCV21) were co-transfected by the same techniques described above except that 1 µg of either the phleomycin resistance vector, pUT507 (for SCV21), or the hygromycin B resistance marker, pY3 (for 4-15, see Blochlinger and Diggelmann, Mol. Cell Biol. 4:2929, 1984), and 10 µg of the xenotropic envelope expression vector, pCMVxeno (above) was used. After selection for transfected cells in the presence of phleomycin or hygromycin, respectively, individual drug resistant cell colonies were expanded and analyzed for intracellular expression of MLV p30<sup>gag</sup> and gp75<sup>env</sup> proteins by Western blot using specific antisera. Clones were identified which expressed relatively high levels of both *gag/pol* and xeno  
10  
15 *env*.

A number of these xenotropic packaging cell lines were tested for their capacity to package retroviral vectors by measuring titre after the introduction of retroviral vectors. The results are presented in Table 5, below.



TABLE 5  
VECTOR TITRE ON XENOTROPIC PCLs

5	CELL CLONE		KT-1 TITRE (CFU/ML) ON HT1080 CELLS
	HT1080	SCV21	$1.0 \times 10^5$
		XF1	$1.0 \times 10^5$
		XF7	$1.0 \times 10^5$
10		XF12 (HX)	$4.5 \times 10^5$
	D17	4-15	
		X6	$9.0 \times 10^4$
		X10 (DX)	$1.3 \times 10^5$
15		X23	$8.0 \times 10^4$

Highest titers are obtained when retroviral vectors are introduced into packaging cell lines by infection, as opposed to transfection (Miller, *et al.*, *Somat. Cell Mol. Genet.*, 12:175, 1986). However, the xenotropic packaging cell lines described herein are blocked for infection by recombinant xenotropic retroviral particles since the cells express a xenotropic *env* protein (*i.e.*, "viral interference"). To overcome the problem of "viral interference," whereby cell lines expressing a xenotropic envelope protein block later infection by xenotropic MLV vectors able to otherwise infect those cell types, vector particles containing other viral envelopes (such as VSV-g protein (Florikiewicz, *et al.*, *J. Cell Bio.* 97:1381, 1983; and Roman, *et al.*, *Exp. Cell Res.* 175:376, 1988) which bind to cell receptors other than the xenotropic receptor) may be generated in the following manner. 10  $\mu$ g of the plasmid DNA encoding the retroviral vector construct to be packaged is co-transfected into a cell line which expresses high levels of *gag/pol* with 10  $\mu$ g of DNA from which a VSV-g protein is expressed. The resultant vector, containing VSV-g protein, is produced transiently in the co-transfected cells. Two days after transfection, cell free supernatants are added to prospective xenotropic packaging cell lines (which express *gag*, *pol*, and *env*). Cell free supernatants are then collected from the confluent monolayers and titered by PCR. Cell clones producing the highest titers are selected as packaging cell lines. This procedure is sometimes referred to "G-hopping."

## VII. Alternative Viral Vector Packaging Techniques

Several additional alternative systems can be used to produce recombinant retrovirus particles carrying a vector construct according to the invention. Some of these systems take advantage of the fact that the insect virus, baculovirus, and the mammalian viruses, vaccinia and adenovirus, have been adapted to make large amounts of any given protein for which the corresponding gene has been cloned. For example, *see* Smith, *et al.* (*Mol. Cell. Biol.* 3:12, 1983); Piccini, *et al.* (*Meth. Enzymology*, 153:545, 1987); and Mansour, *et al.* (*Proc. Natl. Acad. Sci. USA* 82:1359, 1985). These and similar viral vectors can be used to produce proteins in tissue culture cells by insertion of appropriate genes and, hence, could be adapted to make retroviral vector particles.

Adenovirus vectors are derived from nuclear replicating viruses and can be defective. Genes can be inserted into vectors and used to express proteins in mammalian cells either by *in vitro* construction (Ballay, *et al.*, *EMBO J.* 4:3861, 1985) or by recombination in cells (Thummel, *et al.*, *J. Mol. Appl. Genetics* 1:435, 1982).

One preferred method is to construct plasmids using the adenovirus Major Late Promoter (MLP) driving: (1) *gag/pol*, (2) *env*, (3) a modified viral vector construct. A modified viral vector construct is possible because the U3 region of the 5' LTR, which contains the viral vector promoter, can be replaced by other promoter sequences (*see*, for example, Hartman, *Nucl. Acids Res.* 16:9345, 1988). This portion will be replaced after one round of reverse transcriptase by the U3 from the 3' LTR.

These plasmids can then be used to make adenovirus genomes *in vitro* (Ballay, *et al.*, *supra*), which are then transfected into 293 cells (a human cell line making adenovirus E1A protein), for which the adenoviral vectors are defective, to yield pure stocks of *gag/pol*, *env* and retroviral vector carried separately in defective adenovirus vectors. Since the titers of such vectors are typically  $10^7$ - $10^{11}$ /ml, these stocks can be used to infect tissue culture cells simultaneously at high multiplicity. The cells will then be programmed to produce retroviral proteins and retroviral vector genomes at high levels. Since the adenovirus vectors are defective, no large amounts of direct cell lysis will occur and retroviral vectors can be harvested from the cell supernatants.

Other viral vectors such as those derived from unrelated retroviral vectors (*e.g.*, RSV, MMTV or HIV) can be used in the same manner to generate vectors from primary cells. In one embodiment, these adenoviral vectors are used in conjunction with primary cells, giving rise to retroviral vector preparations from primary cells.

Another alternative for making recombinant xenotropic retroviral particles is an *in vitro* packaging system. For example, such a system can employ the following components:

1. *gag/pol* and *env* proteins made in the baculovirus system in a similar manner as described in Smith, *et al.*, *supra*, or in other protein production systems, such as yeast or *E. coli*);
2. vector constructs made using T7 or SP6 transcription systems or other suitable *in vitro* RNA-generating system (*see*, for example, Flamant and Sorge, *J. Virol.* 62:1827, 1988);
3. tRNA made as in (2) or purified from yeast or mammalian cells;
4. liposomes (preferably with embedded *env* protein); and
5. cell extract or purified components (typically from mouse cells) to provide *env* processing, and any or other necessary cell-derived functions.

Within this procedure, the components of (1), (2), and (3) are mixed. The *env* protein, cell extract and pre-liposome mix (in a suitable solvent) is then added. In a preferred embodiment, the *env* protein is embedded in the liposomes prior to adding the resulting liposome-embedded *env* to the mixture of (1), (2), and (3). The mix is treated (*e.g.*, by sonication, temperature manipulation, or rotary dialysis) to allow encapsidation of the nascent viral particles with lipid plus embedded *env* protein in a manner similar to that for liposome encapsidation of pharmaceuticals, as described in Gould-Fogerite, *et al.*, *Anal. Biochem.* 148:15, 1985). This procedure allows the production of high titers of replication incompetent recombinant retroviruses without contamination with pathogenic retroviruses or replication-competent retroviruses.

#### D. Detection of Replication Competent Retroviruses (RCR)

The propensity of the packaging cells described above to generate replication competent retrovirus may be stringently tested by a variety of methods, two of which are described below.

##### i. The Extended S<sup>+</sup>L<sup>-</sup> Assay

The extended S<sup>+</sup>L<sup>-</sup> assay determines whether replication competent, infectious virus is present in the supernatant of the cell line of interest. The assay is based on the empirical observation that infectious retroviruses generate foci on the indicator cell line MiCl<sub>1</sub> (ATCC No. CCL 64.1). The MiCl<sub>1</sub> cell line is derived from the Mv1Lu mink cell line (ATCC No.

CCL 64) by transduction with Murine Sarcoma Virus (MSV). It is a non-producer, non-transformed, revertant clone containing a replication defective murine sarcoma provirus, S<sup>+</sup>, but not a replication competent murine leukemia provirus, L<sup>-</sup>. Infection of MiCl<sub>1</sub> cells with replication competent retrovirus "activates" the MSV genome to trigger "transformation" which results in foci formation.

Supernatant is removed from the cell line to be tested for presence of replication competent retrovirus and passed through a 0.45 µm filter to remove any cells. On day 1, Mv1Lu cells are seeded at 1.0 x 10<sup>5</sup> cells per well (one well per sample to be tested) on a 6 well plate in 2 mL Dulbecco's Modified Eagle Medium (DMEM), 10% FBS and 8 µg/mL polybrene. Mv1Lu cells are plated in the same manner for positive and negative controls on separate 6 well plates. The cells are incubated overnight at 37°C, 10% CO<sub>2</sub>. On day 2, 1.0 mL of test supernatant is added to the Mv1Lu cells. The negative control plates are incubated with 1.0 mL of media. The positive control consists of three dilutions (200 focus forming units (ffu), 20 ffu and 2 ffu each in 1.0 mL media) of MA virus (Miller, *et al.*, *Molec. and Cell Biol.*, 5:431, 1985) which is added to the cells in the positive control wells. The cells are incubated overnight. On day 3, the media is aspirated and 3.0 mL of fresh DMEM and 10% FBS is added to the cells. The cells are allowed to grow to confluency and are split 1:10 on day 6 and day 10, amplifying any replication competent retrovirus. On day 13, the media on the Mv1Lu cells is aspirated and 2.0 mL DMEM and 10% FBS is added to the cells. In addition, the MiCl<sub>1</sub> cells are seeded at 1.0 x 10<sup>5</sup> cells per well in 2.0 mL DMEM, 10% FBS and 8 µg/mL polybrene. On day 14, the supernatant from the Mv1Lu cells is transferred to the corresponding well of the MiCl<sub>1</sub> cells and incubated overnight at 37°C, 10% CO<sub>2</sub>. On day 15, the media is aspirated and 3.0 mL of fresh DMEM and 10% FBS is added to the cells. On day 21, the cells are examined for focus formation (appearing as clustered, refractile cells that overgrow the monolayer and remain attached) on the monolayer of cells. The test article is determined to be contaminated with replication competent retrovirus if foci appear on the MiCl<sub>1</sub> cells.

ii. Cocultivation of Producer Lines and MdH Marker Rescue Assay

As an alternate method to test for the presence of RCR in a retroviral particle producing cell line, producer cells are cocultivated with an equivalent number of *Mus dunni* cells (NIH NIAID Bethesda, MD). Small scale co-cultivations are performed by mixing of 5.0 x 10<sup>5</sup> *Mus dunni* cells with 5.0 x 10<sup>5</sup> producer cells and seeding the mixture into 10 cm plates (10 mL standard culture media/plate, 4 µg/mL polybrene) at day 0. Every 3-4 days

the cultures are split at a 1:10 ratio and  $5.0 \times 10^5$  *Mus dunni* cells are added to each culture plate to effectively dilute out the producer cell line and provide maximum amplification of RCR. On day 14, culture supernatants are harvested, passed through a  $0.45 \mu\text{m}$  cellulose-acetate filter, and tested in the MdH marker rescue assay. Large scale co-cultivations are performed by seeding a mixture of  $1.0 \times 10^8$  *Mus dunni* cells and  $1.0 \times 10^8$  producer cells into a total of twenty T-150 flasks (30 mL standard culture media/flask,  $4 \mu\text{g/mL}$  polybrene). Cultures are split at a ratio of 1:10 on days 3, 6, and 13 and at a ratio of 1:20 on day 9. On day 15, the final supernatants are harvested, filtered and a portion of each is tested in the MdH marker rescue assay.

The MdH marker rescue cell line is cloned from a pool of *Mus dunni* cells transduced with LHL, a retroviral vector encoding the hygromycin B resistance gene (Palmer, *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 84:1055, 1987). The retroviral vector can be rescued from MdH cells upon infection of the cells with RCR. One mL of test sample is added to a well of a 6-well plate containing  $1 \times 10^5$  MdH cells in 2 mL standard culture medium (DMEM with 10% FBS, 1% 200 mM L-glutamine, 1% non-essential amino acids) containing  $4 \mu\text{g/mL}$  polybrene. Media is replaced after 24 hours with standard culture medium without polybrene. Two days later, the entire volume of MdH culture supernatant is passed through a  $0.45 \mu\text{m}$  cellulose-acetate filter and transferred to a well of a 6-well plate containing  $5.0 \times 10^4$  *Mus dunni* target cells in 2 mL standard culture medium containing polybrene. After 24 hours, supernatants are replaced with standard culture media containing  $250 \mu\text{g/mL}$  of hygromycin B and subsequently replaced on days 2 and 5 with media containing  $200 \mu\text{g/mL}$  of hygromycin B. Colonies resistant to hygromycin B appear and are visualized on day 9 post-selection, by staining with 0.2% Coomassie blue.

## EXAMPLE 5

### Production of Recombinant Retroviral Particles

The production of recombinant xenotropic retroviral particles carrying vector constructs according to the invention, representative examples of which are described above, from the human xenotropic and canine xenotropic packaging cell lines HX and DX, respectively, is described below.

A. Transient Plasmid DNA Transfection of Packaging Cell Lines HX and DX

The packaging cell line HX or DX is seeded at  $5.0 \times 10^5$  cells on a 10 cm tissue culture dish on day 1 with DMEM and 10% fetal bovine serum (FBS). On day 2, the media is replaced with 5.0 mL fresh media 4 hours prior to transfection. Standard calcium phosphate-DNA co-precipitations are performed by mixing 40.0  $\mu$ l 2.5 M  $\text{CaCl}_2$ , 10  $\mu$ g of the plasmid encoding the vector to be packaged, and deionized  $\text{H}_2\text{O}$  to a total volume of 400  $\mu$ l. The DNA- $\text{CaCl}_2$  solutions are then added dropwise with constant agitation to 400  $\mu$ l of precipitation buffer (50 mM HEPES-NaOH, pH 7.1; 0.25 M NaCl and 1.5 mM  $\text{Na}_2\text{HPO}_4$ - $\text{NaH}_2\text{PO}_4$ ). These mixtures are incubated at room temperature for 10 minutes. The resultant fine precipitates are added to different culture dishes of cells. The cells are incubated with the DNA precipitate overnight at  $37^\circ\text{C}$ . On day 3, the media is aspirated and fresh media is added. Supernatants are removed on day 4, passed through 0.45  $\mu$ m filters, and stored at  $-80^\circ\text{C}$ .

B. Packaging Cell Line Transduction

DX or HX packaging cells are seeded at  $1.0 \times 10^5$  cells/3 cm tissue culture dish in 2 mL DMEM and 10% FBS, 4  $\mu$ g/mL polybrene (Sigma, St. Louis, MO) on day 1. On day 2, 3.0 mL, 1.0 mL and 0.2 mL of each of a freshly collected supernatant containing VSV-g pseudotyped retroviral particles carrying the desired vector are added to the HX cells. The cells are incubated overnight at  $37^\circ\text{C}$ . On day 3, the pools of cells are cloned by limiting dilution by removing the cells from the plate and counting the cell suspension, diluting the cells suspension down to 10 cells/mL and adding 0.1 mL to each well (1 cell/well) of a 96 well plate (Corning, Corning, NY). Cells are incubated for 14 days at  $37^\circ\text{C}$ , 10%  $\text{CO}_2$ . Several clones producing the desired recombinant xenotropic retrovirus are selected and expanded up to 24 well plates, 6 well plates, and finally to 10 cm plates, at which time the clones are assayed for expression of the appropriate retroviral vector and the supernatants are collected and assayed for retroviral titer.

Using the procedures above, DX and HX cell lines may be derived that produce recombinant xenotropic retroviral vectors with titers greater than or equal to  $1 \times 10^6$  cfu/mL in culture.

### C. Titer Assays

Normally vector titers are determined by transduction of target cells such as HT1080, with appropriate dilutions of a vector preparation, followed by antibiotic selection and counting of surviving colonies (WO 91/02805). However, recombinant retroviral vectors carrying a desired vector construct may not include a gene coding for a selectable marker, as may be the case when the vector construct encodes a large gene of interest, for instance, full length factor VIII, titrating assays other than those based on selection of drug resistant colonies are required. To this end, antibody and PCR assays, the latter of which is described below, may be employed to determine retroviral vector titer, *i.e.*, the number of infectious particles comprising the retroviral vectors of the invention. While such a PCR assay may be required in the context of a vector lacking a selectable marker, it is understood that such an assay can be employed for any given vector.

To use PCR to amplify sequences unique to the retroviral vectors of the invention, various primers are required. Such primers can readily be designed by those skilled in the art and will depend on the retroviral vector backbone employed and the components thereof, the particular region(s) desired to be amplified, *etc.* Representative examples of particular primer pairs include those specific for LTR sequences, packaging signal sequences or other regions of the retroviral backbone, and also include primers specific for the gene of interest in the vector. Additional advantages in using such a PCR titrating assay include the ability to assay for genome rearrangement, *etc.*

In the practice of the present invention, the PCR titrating assay is performed by growing a known number of HT1080 cells, typically  $1 \times 10^5$  cells, transduced with a retroviral vector capable of directing expression of the gene of interest on 6-well plates for at least 16 hr. before harvest. The retroviral vectors used for these transductions are preferably obtained from cell culture supernatants. One well per plate is reserved for cell counting. Cells from the other wells are lysed and their contents isolated. DNA is prepared using a QIAmp Blood Kit for blood and cell culture PCR (QIAGEN, Inc., Chatsworth, CA). DNAs are resuspended at  $5 \times 10^6$  cell equivalents/mL, where one cell equivalent is equal to the DNA content of one cell.

To calculate titer, a standard curve is generated using DNA isolated from untransduced HT1080 cells (negative control) and HT1080 cells transduced with a known vector and having one copy of that vector per cell genome (positive control), such as may be prepared from packaging cell lines transduced with a retroviral vector encoding a selectable marker, *e.g.*, neomycin resistance. For both the positive and negative controls, DNA is

- 70 -

resuspended at  $5 \times 10^6$  cell equivalents/mL. The standard curve is generated by combining different amounts of the positive and negative control DNA, while keeping the total amount of DNA constant, and amplifying specific sequences therefrom by PCR using primers specific to a particular region of the retroviral vector. A representative group of mixtures for

5 generating a standard curve is:

<u>Tube</u>	<u>100%</u>	<u>75%</u>	<u>50%</u>	<u>25%</u>	<u>10%</u>	<u>5%</u>	<u>0%</u>	<u>Blank</u>
Positive Control ( $\mu$ L)	50	37.5	25	12.5	5	2.5	0	0
Negative Control ( $\mu$ L)	0	12.5	25	37.5	45	47.5	50	0
10 Distilled water ( $\mu$ L)	0	0	0	0	0	0	0	50

5.0  $\mu$ L from each tube is placed into one of eight reaction tubes (duplicates are also prepared), with the remainder being stored at  $-20^{\circ}\text{C}$ . 5.0  $\mu$ L from each sample DNA preparation are placed into their own reaction tubes in duplicate. PCR reactions (50  $\mu$ L total volume) are then initiated by adding 45.0  $\mu$ L of a reaction mix containing the following components per tube to be tested: 24.5  $\mu$ L water, 5  $\mu$ L 10X reaction PCR buffer, 4  $\mu$ L of 25 mM  $\text{MgCl}_2$ , 4  $\mu$ L dNTPs (containing 2.5 mM of each of dATP, dGTP, dCTP, and dTTP), 5  $\mu$ L of primer mix (100 ng of each primer), 0.25  $\mu$ L TaqStart monoclonal antibody (Clontech Laboratories, Inc., Palo Alto, CA), 1.00  $\mu$ L TaqStart buffer (Clontech Labs, Inc.), and 0.25  $\mu$ L AmpliTaq DNA polymerase (Perkin-Elmer, Inc., Norwalk, CN). Just prior to aliquoting the reaction mix to the reaction tubes, 1  $\mu$ L of  $\alpha$ - $^{32}\text{P}$  dCTP (250  $\mu\text{Ci}$ ; 3000 C/mmol, 10 mCi/mL, Amersham Corp., Arlington Heights, IL) is added into the reaction mix. After aliquoting 45.0  $\mu$ L the reaction mix into each of the reaction tubes, the tubes are capped and placed into a thermocycler. The particular denaturation, annealing, elongation times and temperatures, and number of thermocycles will vary depending on size and nucleotide composition of the primer pair used. 20 to 25 amplification thermocycles are then performed. 5  $\mu$ L of each reaction is then spotted on DE81 ion exchange chromatography paper (Whatman, Maidstone, England) and air dried for 10 min. The filter is then washed five times, 100 mL per wash, in 50 mM  $\text{Na}_2\text{PO}_4$ , pH 7, 200 mM NaCl, after which it is air dried and then sandwiched in Saran Wrap. Quantitation is performed on a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA). Filters are typically exposed to a phosphor screen, which stores energy from ionizing radiation, for a suitable period, typically about 120 min. After exposure, the phosphor screen is scanned, whereby light is emitted in proportion to the radioactivity on the original filter. The scanning results are then downloaded and plotted on a log scale as cpm (ordinate) versus percent positive control DNA (abscissa).

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Titers (infectious units/mL) for each sample are calculated by multiplying the number of cells from which DNA was isolated by the percentage (converted to decimal form) determined from the standard curve based on the detected radioactivity, divided by the volume of retroviral vector used to transduce the cells. As will be appreciated by those in the art, other methods of detection, such as colorimetric methods, may be employed to label the amplified products.

#### EXAMPLE 6

##### 10        Large Scale Production of Recombinant Xenotropic Retroviruses

The recombinant retroviruses of the invention can be cultivated in a variety of modes, such as in a batch or continuous mode. In addition, various cell culture technologies can be employed to produce commercial scale quantities of the recombinant retroviruses according to the invention. Several such techniques are described below, although others known to those in the art may likewise be employed.

##### A.        Recombinant Retrovirus Production From Hollow Fiber Cultures

##### 20        i.        Culture Initiation

To initiate a hollow fiber culture, the hollow fiber bioreactor (*e.g.*, HFB; Cellco, Inc., Germantown, MD) is first conditioned for 48 hours prior to seeding by simulating a run condition with 100-200 mL of complete growth media at 37°C. The growth media preferably is that to which the cell line has been adapted. All liquids in the HFB when originally shipped should be aspirated and replaced with the complete growth media. When seeding the bioreactor, the cells should not have been split more than 48 hours earlier and should be in log growth phase at the time of harvest for the seeding of the HFB. The cells typically are harvested by trypsinization and pelleted by centrifugation. The cell pellet is then resuspended in 4 mL of 25% pre-conditioned media and delivered to the extra-capillary space by syringe using the side syringe ports found on the HFB. After seeding the HFB, the cells are allowed to adhere for 20 to 30 minutes before starting the circulation pump. During this time, the media used to condition the HFB is replaced with 100-200 mL of 25% pre-conditioned media. The circulation feed pump is initiated with the starting flow rate set at 25 mL/min. (setting 5 with 2 long pump pins). After 1 hour from the time of switching the

pump on, a one mL sample of media is collected in order to record the initial levels of lactate and ammonia. On a daily schedule, 1 ml samples are collected every 24 hours to assay for the daily production of lactate and ammonia. The initial 100-200 mL of media is exchanged with fresh media when lactate levels begin to reach 2.0 g/L (or the equivalent to 22 mmol/L).

5 The same volume of media is replaced until the culture approaches daily levels of 20 mmol/L. When daily levels of lactate reach 20 mmol/L, the size of the reservoir bottle is increased to a 500 mL bottle containing 500 mL of fresh media. The flow feed rate is then increased to 50 mL/min. when the culture begins to produce 2.2 mmol/day of lactate. When daily 500 mL volumes reach 20 mmol/L of lactate, the original Cellco supplied reservoir feeding cap is

10 exchanged for a larger reservoir cap (Unisyn-vender part #240820) adapted for the Cellco system with the addition of tubing and male luer lock fittings. This reservoir cap will accommodate 2 liter Corning bottles. (To avoid the exchange of reservoir caps during a culture run, initiate the run with a large reservoir cap which can also support smaller bottle sizes.) When daily lactate readings are assayed and recorded, the daily levels of lactate

15 production of the culture can be used to determine when the culture reaches maximum cell density, i.e., when the rate of lactate decreases and levels off.

ii. Seeding Density for the 2X- $\beta$ -gal

20 To establish specific seeding requirements, two hollow fiber runs are performed, one run seeded with a low number of cells, the other seeded with a high number of cells. Progress of each culture is tracked by analyzing the daily glucose consumption and lactate production levels.

25 In this experiment, one HFB was seeded with  $1.3 \times 10^7$  cells (representing the low seed culture), the other with  $1.6 \times 10^8$  cells. Here, the cell line 2x- $\beta$ -GAL<sub>17-14</sub> was able to initiate a good hollow fiber run under both seeding conditions. Initiating a run with fewer cells is primarily convenient for reducing the effort required for generating the number of cells required to start a culture, although fewer cells initially extends the time it takes to reach optimal cell densities, which usually yield the highest titers. 2x- $\beta$ -GAL<sub>17-14</sub> adapted

30 well to hollow fiber culture, eventually requiring daily media changes of 500 mL in order to avoid accumulation of toxic levels of lactate. Plateauing of daily lactate production and drops in peak titer production correlated with maximum cell densities and the relative health of the culture.

35 iii. Optimal Titer Concentrations, Frequency of Harvests and Total

### Harvest Amounts

5  $\beta$ -gal titers for the above experiment were determined from frozen samples on 293 cells assayed 48 or 72 hours after transduction. The transduced cells were stained for  $\beta$ -gal activity and counted on a hemocytometer to yield a titer based on the number of blue cells /mL (BCT/mL). Optimum titers were generally obtained on day 7 of a high seed culture at  $1.8 \times 10^8$  BCT/mL from a 72 hour blue cell titer on 293 cells. A duplicate culture initially seeded at a 10 fold lower seeding density peaked at  $5.2 \times 10^7$  BCT/mL from a 48 hour blue cell titer. Compared to flat stock cultures (from tissue culture dishes or flasks) titered using 10 48 hour blue cell titers on HT1080 cells (calculated to be about  $5 \times 10^6$  BCT/mL), the increase in titer by using hollow fiber systems is approximately ten fold higher. These maximum titers observed were reached prior to hitting 20 mmol/L lactate levels, which appeared to reduce titers produced the following week.

15 Crude supernatants can be harvested every 9 hours with out any loss of titer and three harvests per day should be possible with minimum titre loss. In addition, continuous hollow fiber cultures can be maintained for several weeks. When titers were compared between the low and the high seed culture, there was little differences by day 11 between the two seed cultures, both of which averaged  $4 \times 10^7$  BCT/mL.

20

### EXAMPLE 7

#### Two-Phase Purification of Recombinant Retroviruses

##### A. Concentration of DA/ND-7 recombinant particles

25

1400 ml of media (DMEM containing 5% Fetal Bovine Serum) containing DX/ND-7 vector at a titer of  $1.25 \times 10^6$  cfu/ml is used as starting material. Three hundred milliliters of two-phase partitioning components (PEG-8000 (autoclaved), dextran-sulfate, and NaCl) are added to a final concentration of 6.5% PEG, 0.4% dextran-sulphate, and 0.3 M NaCl. The resultant solution is placed into a two-liter separatory funnel, and left in a cold room for 24 30 hours (including two mixing steps approximately 6 to 16 hours apart).

Following the 24 hour period, the bottom layer (approximately 20 mL) is carefully eluted, and the interphase (approximately 1 mL) is collected in a 15 mL conical FALCON tube. The interphase containing vector is diluted to 10 mL by addition of PBS, and

incubated at 37°C in order to bring the solution to room temperature and destabilize the micelles.

- To one-half of the diluted interphase, KCl is added to a final concentration 0.4 M, and mixed well. The tube is then placed on ice for ten minutes, and spun for 2 minutes at 2,000 rpm in a bench-top centrifuge. The supernatant is removed and filtered through a 0.45  $\mu$ m syringe filter. The other half of the interphase containing vector is separated by S-500 Sephadex chromatography in 1X PBS. The results of these concentration processes, as determined in a BCFU assay, are shown below in Table 6:

10

TABLE 6

<u>PHASE</u>	<u>QUANTITY OF VECTOR</u>
Crude	$1.75 \times 10^9$ bcfu
Separation: Top phase	$1.4 \times 10^8$ bcfu
Separation: Interphase	$7(+/-3) \times 10^8$ bcfu
Separation: Bottom phase	$2 \times 10^6$ bcfu
Final step: KCl separation	$*6(+/-3) \times 10^8$ bcfu
Final step: S-500 separation	$*1.8(+/-0.3) \times 10^8$ bcfu

\* Note that since the sample was split into two halves, that these numbers were doubled in order to represent the level of purification that would be expected if the entire 1 mL interphase was separated as indicated.

- 15 In summary, 1.4 liters of crude research grade supernatant containing recombinant retroviral particles may be reduced to a 10 mL volume, with approximately 50% (+/-20%) being recovered when KCl separation is utilized as the final step. When S-500 chromatography is utilized as the final step, only about 10% of the initial recombinant retroviral particles are recovered in a 14 mL.

- 20 In order to complete concentration of the retroviral vector particles, the vector-containing solution may be further subjected to concentration utilizing an MY-membrane Amicon filter, thereby reducing the volume from 10 to 14 mL, down to less than 1 mL.

EXAMPLE 8Production of Vector from DX/ND7  $\beta$ -gal Clone 87 Utilizing a Cell Factory

5 DX/ND7 bgal clone 87, an expression vector, was grown in cell factories. Cells were grown in DMEM supplemented with Fetal Bovine Serum in roller bottles until enough cells to seed 20 10-layer cell factories (NUNC) at a 1:3 dilution were obtained. Each 10-layer cell factory is seeded with approximately 0.8 liters of cell medium.

10 Cells were seeded into the cell factory by pouring media containing cells into the factory so that the suspensions evenly fill the 10 layers. The factory is then carefully tilted away from the port side to prevent the suspension from redistribution in the common tube. Finally, the cell factory is rotated into its final upright position. A hepa vent filter is attached to each port. The factory was then placed in a CO<sub>2</sub> incubator.

15 In three days, and for each of the next three days, supernatant containing vector was harvested. The cell factory is placed in a tissue culture hood. One filter is removed and sterile transfer tubing is connected to the open port. The factory is lifted so that supernatant drains into the tubing. Approximately 2 liters of supernatant is harvested from each factory. Fresh DMEM/FBS is used to replenish the lost medium. The transfer tubing is removed and the factory replaced in the incubator. From 20 cell factories, approximately 90 liters of crude  
20 vector containing supernatant were obtained. Verification of the vector was performed by transduction of HT1080 cells. These cells were harvested 2 days later and stained for b-gal protein. The titer of the supernatant was determined to be  $2 \times 10^7$ /ml.

25

EXAMPLE 9

## Concentration of Recombinant Retrovirus by Low-Speed Centrifugation

A. Retrovector Supernatant Preparation

30

Producer cell lines DA/ $\beta$ gal and HX/DN-7 were cultured in a culture flask and a roller bottle, respectively, containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum plus 1mM L-Glutamine, Sodium pyruvate, non-essential amino acids and antibiotics. Viral supernatant was harvested from the flask and

roller bottle, and were filtered through a 0.45  $\mu$ m syringe filter. The filtered supernatants were stored either at 4°C (HX/ND7), or frozen at -70°C (DA $\beta$ -gal).

#### B. Virus Concentration

5

Viral supernatant was aliquoted into 50 ml sterile OAKRIDGE screw cap tubes, and placed into an SS34 rotor for use in a Sorvall centrifuge. The tubes were spun for 1 hour at 16,000 rpm (25,000g-force) at 4°C. Upon completion of the spin, the tubes were removed, the supernatant decanted and a small opaque pellet resuspended in the DMEM media

10

described above.

#### C. Virus Titration

15

Concentrated virus was titered on HT1080 cells plated 24 hours earlier at a cell density of  $2 \times 10^5$  cells per well in a six well plate + 4  $\mu$ g/ml polybrene. Briefly, virus preps were diluted from 1/10 to 1/10,000 and 50  $\mu$ l of each dilution was used to infect one well from the six well plate. Plates were incubated overnight at 37°C. Forty-eight hours later, cells were fixed and stained with X-gal. The results are set forth below in Table 7.

20

Table 7. Virus Concentration through Low Speed Centrifugation

<i>Parameter description</i>	<i>Experiment number</i>				
	1	2		3	
Virus source	DA $\beta$ -gal	DA $\beta$ -gal	HX/ND7	DA $\beta$ -gal	HX/ND7
Titer of normal harvest	$4.4 \times 10^6$	$2.1 \times 10^6$	$3.2 \times 10^5$	$5 \times 10^6$	$5 \times 10^5$
Titer of virus concentrate	$6 \times 10^8$	$7.4 \times 10^7$	$3.2 \times 10^7$	$2.9 \times 10^8$	$3.9 \times 10^7$
Starting volume	80 ml	.39 ml	39 ml	118ml	40ml
Final concentrate volume	.5 ml	.36 ml	.36 ml	.78ml	.28ml
Fold virus concentration	136X	34X	100X	58X	78X
Virus recovery	87%	30%	91%	50%	99%

As is evident from Table 7, virus recovery ranged from 30% to 99%, with the best recovery being obtained from human producer cells (HX/ND7; recovery ranged from 91% to 99%).

5

#### EXAMPLE 10

##### Concentration of Recombinant Retroviruses By Ultrafiltration

10 S-500 purified supernatant containing the  $\beta$ -gal expressing recombinant retrovirus DX/CB-bgal and partially concentrated supernatant containing the same virus were each filtered through a 0.45  $\mu$ m filter, and loaded into a CENTRIPREP-100 filter (product #4308, Amicon, MA). The supernatants were kept at a temperature of 4°C throughout this procedure, including during centrifugation. The CENTRIPREP filters were spun three times each for 45 to 60 minutes at 500 x G. Between each spin the filtrate was decanted. The  
15 retentate was thus sequentially reduced, such that the initial 15 mL (or 10 mL) volume was reduced to approximately 0.6 mL per unit.

The resultant titer was determined by assaying HT1080 target cells set up at a concentration of  $1 \times 10^5$  cells per well 24 hours prior to transduction of the viral sample. Cells were transduced in the presence of 8  $\mu$ g/ml polybrene and 2 mL growth media (DMEM plus 10% FBS) per well. As shown in Table 8 below, approximately one hundred percent of  
20 the virus was recovered utilizing this procedure (note that titers are in BCFU/ml).

Table 8

	<i>Pre-centriprep titer/volume.</i>	<i>Final titer/volume</i>
S-500	$4 \times 10^7$ /15 ml	$1.3 \times 10^9$ /0.6 ml
part. conc.	$3 \times 10^8$ /10 ml	$1 \times 10^{10}$ /0.6 ml

25

#### EXAMPLE 11

##### Preparation of Recombinant Retrovirus in a Bioreactor

30 A. Freezing protocol

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Producer cells are frozen in DMEM media containing 10% to 20% FBS, and 5 to 15% DMSO, at a concentration of  $1 \times 10^7$  cells/ml/vial. Cells are frozen in a controlled rate freezer (Series PC, Controlled Rate Freezing System, Custom Biogenic Systems, Warren MI) at a rate of from 1 to  $10^\circ\text{C}$  per minute. Frozen cells are stored in liquid nitrogen.

5

B. Bioreactor protocol

Cells are thawed from frozen vials at  $37^\circ\text{C}$ , washed once with media to remove DMSO, and expanded into  $850\text{ cm}^2$  "FALCON" roller bottles (Corning, Corning, NY). Expanded cell culture is used to inoculate a "CELLIGEN PLUS" bioreactor (5 liter working volume; New Brunswick, Edison, NJ). The cells are grown on microcarriers (*i.e.*, Cytodex 1 or Cytodex 2; Pharmacia, Piscataway, N.J.) at a concentration of 3 to 15 g/L microcarrier. Initial inoculation densities are from 4 to 9 cells/bead at half to full volume for 2 to 24 hours. The media constituents for virus production are DMEM-high glucose (Irvine Scientific, Santa Ana, CA.) basal media supplemented with FBS (10 to 20%), Glutamine (8 to 15mM),  
10 glucose (4.5 to 6.5 g/L), Nonessential amino acids (1X), RPMI 1640 amino acids (0.2 to 9.6X), 10 mM HEPES, RPMI 1640 Vitamins (0.2 to 5X).

During culture, pH (6.9 to 7.6) and dissolved oxygen ("DO" 5 to 90%) are controlled by the use of a four gas system which includes air, oxygen, nitrogen, and carbon dioxide. After several days of batch growth the culture is then continuously perfused with  
15 fresh media with concurrent continuous harvesting in an escalating perfusion rate of 0.5 to 2.5 volumes/day. Cell retention is the result of differential sedimentation of cell covered beads in a decanting column.

During operation the bioreactor is monitored for viable cells, titer, glucose, lactate, ammonia levels, and lack of contamination. Viable cells and titer range from  $1 \times 10^5$  cells/ml  
20 to  $1 \times 10^7$  cells/ml. Glucose ranges from 6 to 0.25 g/L, Lactate from 1 to 25 mM, and Ammonia ranges from 0.5 to 30 mM. Cells are incubated in the bioreactor for 5 to 25 days.

EXAMPLE 12

30

Cell sorting and analysis

Apheresed samples were obtained with informed consent from multiple myeloma patients treated at the University of Arkansas Medical Center. The patients were treated on day 1 with cyclophosphamide at  $6\text{ g/m}^2$  ( $1.5\text{ g/m}^2$  every 3 hours x 4 doses). From day 1 until  
35 the start of leukopheresis (usually 10-28 days), granulocyte macrophage colony stimulating



factor (GM-CSF) was given at 0.25 mg/m<sup>2</sup>/day. Apheresis for total white cells was started when the peripheral blood white cell count was greater than 500 cells/ml and the platelet count was greater than 50,000 cells/ml. Patients were apheresed daily until from  $6 \times 10^8$  mononuclear cells (MNC) were collected.

5       Antibodies to CD14 and CD15 were obtained as FITC conjugates from Becton-Dickinson. Antibody to Thy-1 (GM201) was obtained from Dr. Wolfgang Retig (Ludwig Institute, New York), and was detected with anti- $\gamma$ 1-PE conjugate from Caltag. Antibody to CD34 (Tük 3) was obtained from Dr. Andreas Ziegler (University of Berlin), and detected with an anti- $\gamma$ 3-Texas Red conjugate (Southern Biotechnologies).

10       For cell sorting, fresh MPB samples were elutriated with a JE5.0 Beckman counterflow elutriator equipped with a Sanderson chamber (Beckman, Palo Alto, CA). Cells were resuspended in elutriation medium (Biowhittaker, Walkersville, MD) at pH 7.2, supplemented with 0.5% human serum albumin (HSA). The rotor speed was set at 2000 RPM, the cells were introduced, and the first fraction collected at a flow rate of 9.6 ml/min.

15       Fractions 2 and 3 were collected at the respective flow rates of 14 and 16 ml/min. The larger cells remaining in the chamber were collected after stopping the rotor. Cells were resuspended in RPMI supplemented with 5% HSA, 10  $\mu$ g/ml DNase I and penicillin/streptomycin at 50 U/ml and 50  $\mu$ g/ml, respectively. Fractions 2 and 3 were pooled and incubated with 1 mg/ml heat-inactivated human gamma-globulin to block non-specific Fc binding. Granulocytes were further depleted by incubation with CD15

20       conjugated to magnetic beads (Dynal M450, Oslo, Norway) followed by magnetic selection.

      Anti-CD34 antibody or an IgG3 isotype matched control were added to cells in staining buffer (HBSS, 2% FCS, 10 mM HEPES) for 20 minutes on ice, together with anti-Thy-1 antibody at 5 mg/ml. Cells were washed with a FCS underlay, and then incubated

25       with Texas Red conjugated goat anti-mouse IgG3 antibody and phycoerythrin-conjugated goat anti-mouse IgG1 antibody for 20 minutes on ice. Blocking IgG1 was then added for 10 minutes. After blocking, the FITC-conjugated lineage antibody panel (CD14 and CD15) was added, and incubated for another 20 minutes on ice. After a final washing, cells were resuspended in staining buffer containing propidium iodide (PI).

30       Cells were sorted either on the FACSTAR Plus cell sorter equipped with dual argon ion lasers, the primary laser emitting at 488 nm and a dye laser (Rhodamine 6G) emitting at 600 nm (Coherent Innova 90, Santa Cruz, CA) or on a high speed cell sorter as described in PCT patent application number PCT/US93/08205. Residual erythrocytes, debris and dead cells were excluded by light scatter gating plus an FL3 (PI) low gate. Following isolation of

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a cell population by flow cytometry, the sample was diluted 1:1 in HBSS, pelleted, and resuspended in HBSS for hemocytometer counting.

### EXAMPLE 13

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#### Transduction

1 x 10<sup>5</sup> CD34<sup>+</sup>Thy<sup>+</sup>Lin<sup>-</sup> (MPB) viable cells obtained as described above, were suspended in 1 ml of freshly thawed retroviral supernatant with cytokines at the following concentrations: c-kit ligand (Amgen) 100 ng/ml; IL-3 (Sandoz) 25 ng/ml; IL-6 (Sandoz) 50  
10 ng/ml. Protamine sulfate was added at a final concentration of 4 ug/ml. At 24 and 48 hours, supernatant was replaced with freshly thawed retroviral supernatant. Cytokines and protamine sulfate were added at the concentrations listed above. After 72 hours, cells were harvested and placed in assays to determine transduction frequency. As a control, cells were  
15 cultured DMEM with cytokines and protamine sulfate as described above, but without retroviral supernatant.

### EXAMPLE 14

#### Methylcellulose assay

20 In order to determine transduction frequency of the stem cells, the following experiment was performed. 5 x 10<sup>3</sup> or 2.5 x 10<sup>3</sup> cells from each transduction were added to 5 ml of methylcellulose (Stem Cell Technologies) containing the following cytokines: c-kit ligand 10 ng/ml; GM-CSF 25 ng/ml; G-CSF 25 ng/ml; IL-3 10 ng/ml; rhEPO 2 units/ml. 1.1  
25 ml of the cell/cytokine methylcellulose mixture was plated onto four 3 cm gridded plates using a 5 ml syringe and 16.5 gauge needle, and the plates were placed in a 37°C incubator for 2 weeks.

After 14 days, single methylcellulose colonies were picked and suspended in 50 µl Lysing Buffer (75 mM KCl, 10 mM Tris-HCl pH 9.25, 1.5 mM MgCl<sub>2</sub>, 0.5% Tween 20, 0.5% NP40, 1 mg/ml proteinase K) for PCR analysis.

30 The lysates were amplified by PCR to determine the presence of the vector in the transduced cells. The PCR assay amplified a 134 bp fragment of the vector psi packaging sequence. A 40 cycle amplification (25 ul total volume) in Perkin-Elmer 9600 Cycler using 10 ul lysate and 0.20 uM each F1 and B5 primers (Genset) was performed as follows: one cycle 95°C 30 sec.; 40 cycles 95°C 10 sec., 64°C 15 sec., 72°C 15 sec.; final extension 72°C

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5 min. PCR products were visualized on ethidium bromide agarose gels. The results appear in Table 9.

TABLE 9

Experiment	Tissue	Retrovirus (Batch)	Titer	n	134 Psi Colonies	Transduced Frequency
1	MF-7	Xeno (8/18/94)	$1.0 \times 10^7$	90	69	77%
1	MF-7	DMEM Mock		20	1	5%
2	MF-13	Xeno (8/18/94)	$1.8 \times 10^6$	80	5	6%
2	MF-13	Xeno (8/18/94)	$1.0 \times 10^7$	86	41	48%
3	#9748	Xeno (8/18/94)	$1.0 \times 10^7$	86	48	56%
4	MF-20	Xeno (8/18/94)	$1.8 \times 10^6$	81	25	31%
4	MF-20	Xeno (8/18/94)	$1.0 \times 10^7$	81	39	48%
5	MF-27	Xeno (8/18/94)	$1.8 \times 10^6$	81	5	6%
5	MF-27	Xeno (8/18/94)	$1.0 \times 10^7$	81	25	31%
6	MF-30	Xeno (8/18/94)	$1.8 \times 10^6$	81	3	4%
6	MF-30	Xeno (8/18/94)	$1.0 \times 10^7$	81	35	43%
7	MF-13	Xeno (8/18/94)	$1.0 \times 10^7$	70	18	26%
8	MF-10	Xeno (8/18/94)	$1.0 \times 10^7$	70	19	27%

Example 15

## LTCIC Transduction Assay

CD34+Thy+Lin- (MPB) viable cells were counted and transduced for 72 hours as described in Example 13 herein. Viable cells seeded in 2-fold serial dilutions in a 96- well plate on pre-formed AC6.21 monolayers in Whitlock-Witte media (50/50 RPMI/IMDM,

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10% Fetal Calf Serum, Pen-Strep. L-Glutamine, Sodium Pyruvate, and 2-ME) supplemented with LIF at 50 ng/ml and IL-6 at 10 ng/ml. Final cell concentrations, set up in duplicate, were 100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 cells/well respectively. The plates were cultured at 37°C, 5% CO<sub>2</sub> for 5 weeks, with the cells fed weekly by replacement of one-half  
5 the spent media with fresh media and cytokines.

Bulk LTCIC Assay:

After 5 weeks, plates were scored for cobblestone area forming cells (CAFC).  
10 LTCIC plates were prepared by harvesting wells containing multiple cobblestones and filtering the cultures through Nitex filters into 24-well plates. Cultures were overlaid with approximately 0.6 mls methylcellulose containing 10 ng/ml c-kit ligand, 25 ng/ml GM-CSF, 25 ng/ml G-CSF, 10 ng/ml IL-3, and 1.2 U/ml rhEPO, and cultured for 10-14 days at 37°C, 5% CO<sub>2</sub> (i.e., approximately 7 weeks culture from the time of transduction). Individual  
15 methylcellulose colonies were picked and analyzed by PCR for the presence of the vector psi packaging sequence as described in Example 14 herein. The results are shown in Table 10 below. The numbers represent the number of bulk LTCIC wells with at least one PCR positive colony/total number of bulk wells analyzed by PCR. The results show that the xenotropic vector did transduce stem cells as measured by LTCIC. The numbers do not  
20 represent a transduction frequency of LTCIC since the wells that were harvested contained multiple cobblestone areas, and the resulting methylcellulose colonies could have been derived from different cobblestone area forming cells and hence LTCIC.

Clonal LTCIC Assay:

25

The clonal LTCIC assay was performed as described above for the bulk LTCIC assay with the exception that individual wells were harvested where, based on limiting dilution, the wells harvested were most likely clonal (>95% probability based on Poisson distribution, i.e., <37% of the wells at a given dilution contained a CAFC).

The results are shown in Table 10 below. The numbers represent the number of clonogenic LTCIC wells with at least one positive colony by PCR/total number of clonal wells analyzed by PCR. The results from one experiment show that the xenotropic vector transduced 2 of 9 LTCIC. Since the cells from each well harvested were derived from a single cell, the results of the clonal LTCIC assay represents a true LTCIC transduction frequency.

TABLE 10

Experiment	Retrovirus	Titer	Bulk LTC-IC*	Clonal LTC-IC**
1.	Xeno	1.8e6	0/4	ND
2.	Xeno	1.8e6	0/3	ND
3.	Xeno	1.8e6	2/12	2/9
4.	Xeno	1e7	1/7	ND

10

\* Numbers indicate no. bulk LTCIC wells with at least one psi(+) positive colony/total number of bulk wells analyzed by PCR.

15

\*\* Numbers indicate no. clonogenic LTCIC wells with at least one psi(+) positive colony/total number clonal wells analyzed by PCR.

20

While the present invention has been described above both generally and in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art in light of the description, *supra*. Therefore, it is intended that the appended claims cover all such variations coming within the scope of the invention as claimed.

25

Additionally, the publications and other materials cited to illuminate the background of the invention, and in particular, to provide additional details concerning its practice as described in the detailed description and examples, are hereby incorporated by reference in their entirety.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANTS: CHIRON VIAGENE, INC.  
SYSTEMIX
- (ii) TITLE OF INVENTION: High Efficiency Ex Vivo Transduction of  
Hematopoietic Stem Cells by Recombinant Xenotropic  
Retroviral Preparations
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Chiron Viagene, Inc.
  - (B) STREET: 4560 Horton Street
  - (C) CITY: Emeryville
  - (D) STATE: California
  - (E) COUNTRY: U.S.A.
  - (F) ZIP: 94608
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: Unassigned
  - (B) FILING DATE: 19 April 1996
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Kruse, Norman J.
  - (B) REGISTRATION NUMBER: 35,235
  - (C) REFERENCE/DOCKET NUMBER: 1156.100
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (510) 601-3520
  - (B) TELEFAX: (510) 655-3542

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAATAAATAG ATTTAGATTT A

21

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCCTCGAGAC GATGAAATAT ACAAGTTATA TCTTG

35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATCGATCC ATTACTGGGA TGCTCTTCGA CCTGG

35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCCTCGAGCT CGAGCGATGA AATATAACAAG TTATATCTTG

40

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTCATCTCGT TTCTTTTGT TGCTATT

27

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AATAGCAACA AAAAGAAACG AGATGAC

27

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 40 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCATCGATAT CGATCATTAC TGGGATGCTC TTCGACCTCG

40

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATAAATAGAA GGCCTGATAT G

21

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCCTCGAGAC AATGTACAGG ATGCAACTCC TGTCT

35

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs



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(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAATCGATTT ATCAAGTCAG TGTTGAGATG ATGCT

35

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CLAIMS**WE CLAIM:**

1. A method of producing transduced hematopoietic stem cells, the method comprising:
  - (a) obtaining a population of hematopoietic stem cells from a patient; and
  - (b) transducing the population of hematopoietic stem cells with recombinant retroviral particles substantially free from contamination with replication competent retrovirus, wherein the recombinant retroviral particles carry a vector construct encoding a gene of interest.
2. The method of Claim 1 wherein the gene of interest encodes a protein or an active portion of a protein selected from the group consisting of a cytokine, a colony stimulating factor, a clotting factor, and a hormone.
3. The method of claim 1 wherein said recombinant retroviral particle is a xenotropic recombinant retroviral particle.
4. The method of claim 1 wherein said hematopoietic stem cell is a CD34+Thy-1+Lin- hematopoietic stem cell.
5. The method of claim 1 wherein said recombinant retroviral vector particles are a high titer preparation of recombinant retroviral particles.
6. A composition comprising a population of hematopoietic stem cells transduced with recombinant retroviral particles substantially free from contamination with replication competent retrovirus, wherein the recombinant retroviral particles carry a vector construct encoding a gene of interest.

7. The composition of Claim 6 wherein the gene of interest encodes a protein or active portion of a protein selected from the group consisting of a cytokine, a colony stimulating factor, a clotting factor, and a hormone.
8. The composition of Claim 7 wherein said cytokine is selected from the group consisting of IL-2 and gamma-interferon.
9. The composition of Claim 6 wherein said recombinant retroviral particle is a xenotropic recombinant retroviral vector particle.
10. The composition of Claim 6 wherein said hematopoietic stem cell is a CD34+Thy-1+Lin- hematopoietic stem cell.
11. The composition of claim 6 wherein said hematopoietic stem cells are transduced with a high titer preparation of hematopoietic stem cells.
12. A hematopoietic stem cell transduced with a recombinant retroviral particle substantially free from contamination with replication competent retrovirus, wherein the recombinant retroviral particle carries a vector construct encoding a gene of interest.
13. The hematopoietic stem cell of Claim 12 wherein the gene of interest construct encodes a protein or active portion of a protein selected from the group consisting of a cytokine, a colony stimulating factor, a clotting factor, and a hormone.
14. The hematopoietic stem cell of claim 12 wherein said hematopoietic stem cell is a CD34+Thy-1+Lin- hematopoietic stem cell.
15. The hematopoietic stem cell of claim 12 wherein said recombinant retroviral vector particle is a xenotropic hematopoietic stem cell.

16. The hematopoietic stem cell of claim 15 wherein said hematopoietic stem cell is transduced by a high titer preparation of recombinant retroviral particles.
17. A method of treating a patient having a genetic disease, the method comprising:
- (a) obtaining a population of hematopoietic stem cells from the patient;
  - (b) transducing the population of hematopoietic stem cells with recombinant retroviral particles substantially free from contamination with replication competent retrovirus, wherein the recombinant retroviral particles carry a vector construct encoding a gene of interest useful in treating the genetic disease; and
  - (c) re-introducing into the patient a therapeutically effective amount of the population of transduced hematopoietic stem cells.
18. The method of claim 17 wherein said population of hematopoietic stem cells is transduced by a high titer preparation of recombinant retroviral particles.
19. The method of claim 17 wherein said hematopoietic stem cells are CD34+Thy-1+Lin- hematopoietic stem cells.
20. The method of claim 17 wherein said recombinant retroviral vector particles are xenotropic recombinant retroviral vector particles.
21. A method of treating a patient having cancer, the method comprising:
- (a) obtaining a population of hematopoietic stem cells from the patient;
  - (b) transducing the population of hematopoietic stem cells with recombinant retroviral particles substantially free from contamination with replication competent retrovirus, wherein the recombinant retroviral particles carry a vector construct encoding a gene of interest useful in treating cancer; and
  - (c) re-introducing into the patient a therapeutically effective amount of the population of transduced hematopoietic stem cells.
22. The method of claim 21 wherein said population of hematopoietic stem cells is transduced by a high titer preparation of recombinant retroviral particles.

23. The method of claim 21 wherein said hematopoietic stem cells are CD34+Thy-1+Lin-hematopoietic stem cells.
24. The method of claim 21 wherein said recombinant retroviral vector particles are ~~xenotropic recombinant retroviral vector particles~~.
25. A method of treating a patient having an infectious disease, the method comprising:  
(a) obtaining a population of hematopoietic stem cells from the patient;  
(b) transducing the population of hematopoietic stem cells with recombinant retroviral particles substantially free from contamination with replication competent retrovirus, wherein the recombinant retroviral particles carry a vector construct encoding a gene of interest useful in treating the infectious disease; and  
(c) re-introducing into the patient a therapeutically effective amount of the population of transduced hematopoietic stem cells.
26. The method of claim 25 wherein said population of hematopoietic stem cells is transduced by a high titer preparation of recombinant retroviral particles.
27. The method of claim 25 wherein said hematopoietic stem cells are CD34+Thy-1+Lin-hematopoietic stem cells.
28. The method of claim 25 wherein said recombinant retroviral vector particles are xenotropic recombinant retroviral vector particles.
29. A method of treating a patient having a degenerative disease, the method comprising:  
(a) obtaining a population of hematopoietic stem cells from the patient;  
(b) transducing the population of hematopoietic stem cells with recombinant retroviral particles substantially free from contamination with replication competent retrovirus, wherein the recombinant retroviral particles carry a vector construct encoding a gene of interest useful in treating the degenerative disease; and  
(c) re-introducing into the patient a therapeutically effective amount of the population of transduced hematopoietic stem cells.

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30. The method of claim 29 wherein said population of hematopoietic stem cells is transduced by a high titer preparation of recombinant retroviral particles.
31. The method of claim 29 wherein said hematopoietic stem cells are CD34+Thy-1+Lin-~~hematopoietic~~ hematopoietic stem cells.
32. The method of claim 29 wherein said recombinant retroviral vector particles are xenotropic recombinant retroviral vector particles.
33. A method of treating a patient having an inflammatory disease, the method comprising:
- (a) obtaining a population of hematopoietic stem cells from the patient;
  - (b) transducing the population of hematopoietic stem cells with recombinant retroviral particles substantially free from contamination with replication competent retrovirus, wherein the recombinant retroviral particles carry a vector construct encoding a gene of interest useful in treating the inflammatory disease; and
  - (c) re-introducing into the patient a therapeutically effective amount of the population of transduced hematopoietic stem cells.
34. The method of claim 33 wherein said population of hematopoietic stem cells is transduced by a high titer preparation of recombinant retroviral particles.
35. The method of claim 33 wherein said hematopoietic stem cells are CD34+Thy-1+Lin- hematopoietic stem cells.
36. The method of claim 33 wherein said recombinant retroviral vector particles are xenotropic recombinant retroviral vector particles.
37. A method of treating a patient having a cardiovascular disease, the method comprising:
- (a) obtaining a population of hematopoietic stem cells from the patient;
  - (b) transducing the population of hematopoietic stem cells with recombinant

retroviral particles substantially free from contamination with replication competent retrovirus, wherein the recombinant retroviral particles carry a vector construct encoding a gene of interest useful in treating the cardiovascular disease; and

(c) re-introducing into the patient a therapeutically effective amount of the population of transduced hematopoietic stem cells.

38. The method of claim 37 wherein said population of hematopoietic stem cells is transduced by a high titer preparation of recombinant retroviral particles.

39. The method of claim 37 wherein said hematopoietic stem cells are CD34+Thy-1+Lin-hematopoietic stem cells.

40. The method of claim 37 wherein said recombinant retroviral vector particles are xenotropic recombinant retroviral vector particles.

41. A method of treating a patient having an autoimmune disease, the method comprising:

- (a) obtaining a population of hematopoietic stem cells from the patient;
- (b) transducing the population of hematopoietic stem cells with recombinant xenotropic retroviral particles substantially free from contamination with replication competent retrovirus, wherein the recombinant xenotropic retroviral particles carry a vector construct encoding a gene of interest useful in treating the autoimmune disease; and
- (c) re-introducing into the patient a therapeutically effective amount of the population of transduced hematopoietic stem cells.

42. The method of claim 41 wherein said population of hematopoietic stem cells is transduced by a high titer preparation of recombinant retroviral particles.

43. The method of claim 41 wherein said hematopoietic stem cells are CD34+Thy-1+Lin-hematopoietic stem cells.

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44. The method of claim 41 wherein said recombinant retroviral vector particles are xenotropic recombinant retroviral vector particles.

45. The method according to any one of claims 17, 21, 25, 29, 33, 37 or 44 further comprising expanding the transduced population of hematopoietic stem cells *in vitro* prior to re-introduction of the cells into the patient.



## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 96/05432

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C12N5/10 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HUMAN GENE THERAPY, vol. 4, no. 3, 1 June 1993, pages 239-247, XP000574386 VAN BEUSECHEM V W ET AL: "RETROVIRUS-MEDIATED GENE TRANSFER INTO RHESUS MONKEY HEMATOPOIETIC STEM CELLS: THE EFFECT OF VIRAL TITERS ON TRANSDUCTION EFFICIENCY" see the whole document ---	1-45
X	WO,A,93 18137 (SYSTEMIX, INC.) 16 September 1993 see page 33 - page 36 ---	1-45
	-/--	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\*A\* document member of the same patent family

Date of the actual completion of the international search

12 September 1996

Date of mailing of the international search report

27.09.96

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 96/05432

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>THROMBOSIS AND HAEMOSTASIS, vol. 67, no. 3, 2 March 1992, pages 341-345, XP000570193 HOEBEN R C ET AL: "TOWARD GENE THERAPY IN HAEMOPHILIA A: RETROVIRUS-MEDIATED TRANSFER OF A FACTOR VIII GENE INTO MURINE HAEMATOPOIETIC CELLS" see the whole document ---</p>	1-45
X	<p>ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, 1 January 1990, pages 415-426, XP000574377 BODINE D M ET AL: "DEVELOPMENT OF A HIGH-TITER RETROVIRUS PRODUCER CELL LINE AND HEMATOPOIETIC STEM CELLS STRATEGIES FOR RETROVIRUS-MEDIATED GENE TRANSFER INTO RHESUS MONKEY" see the whole document ---</p>	1-45
X	<p>WO,A,93 11230 (DYNAL AS) 10 June 1993 see example 2 -----</p>	1-45

# INTERNATIONAL SEARCH REPORT

In national application No.

PCT/US 96/05432

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 17-45  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 17-45 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/05432

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9318137	16-09-93	CA-A- 2131368 EP-A- 0631618 JP-T- 7504331	16-09-93 04-01-95 18-05-95
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WO-A-9311230	10-06-93	AU-A- 3084492	28-06-93
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